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Behavioral/Cognitive

Neuropeptidergic Signaling Partitions Arousal Behaviors in Zebrafish

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Animals modulate their arousal state to ensure that their sensory responsiveness and locomotor activity match environmental demands. Neuropeptides can regulate arousal, but studies of their roles in vertebrates have been constrained by the vast array of neuropeptides and their pleiotropic effects. To overcome these limitations, we systematically dissected the neuropeptidergic modulation of arousal in larval zebrafish. We quantified spontaneous locomotor activity and responsiveness to sensory stimuli after genetically induced expression of seven evolutionarily conserved neuropeptides, including adenylate cyclase activating polypeptide 1b (adcyap1b), cocaine-related and amphetamine-related transcript (cart), cholecystokinin (cck), calcitonin gene-related peptide (cgrp), galanin, hypocretin, and nociceptin. Our study reveals that arousal behaviors are dissociable: neuropeptide expression uncoupled spontaneous activity from sensory responsiveness, and uncovered modality-specific effects upon sensory responsiveness. Principal components analysis and phenotypic clustering revealed both shared and divergent features of neuropeptidergic functions: hypocretin and cgrp stimulated spontaneous locomotor activity, whereas galanin and nociceptin attenuated these behaviors. In contrast, cart and adcyap1b enhanced sensory responsiveness yet had minimal impacts on spontaneous activity, and cck expression induced the opposite effects. Furthermore, hypocretin and nociceptin induced modality-specific differences in responsiveness to changes in illumination. Our study provides the first systematic and high-throughput analysis of neuropeptidergic modulation of arousal, demonstrates that arousal can be partitioned into independent behavioral components, and reveals novel and conserved functions of neuropeptides in regulating arousal.

Introduction

Arousal is fundamental to life, from the vigilance required to hunt prey or avoid predators, to the drive needed to obtain sustenance and mates. Defects in arousal can be debilitating. For the ~15–20% of Americans with sleep disorders, approximately half exhibit insomnia, which is associated with an inability to regulate physiological arousal (Mahowald and Schenk, 2005; Colten and Altevogt, 2006; Saper et al., 2010). Inappropriately elevated arousal is associated with stress, anxiety, and hyperactivity, whereas abnormally low arousal can cause inattention, excessive sleepiness, chronic fatigue, and vegetative states (Pfaff and Banavar, 2007; Pfaff et al., 2008; Berridge et al., 2010). Nearly a century of research has elucidated the primary arousal-promoting neuroanatomy: ascending projections from brainstem nuclei stimulate wakefulness in the brain (von Economo, 1930; Moruzzi and Magoun, 1949; Saper et al., 2005, 2010; Pfaff and Banavar, 2007; Fuller et al., 2011).

Despite extensive studies of the arousal systems, several important questions remain. For example, the partitioning of arousal into individual behavioral components, including spontaneous locomotor activity and sensory responsiveness, has stimulated debate regarding the independence of different arousal behaviors (Robbins, 1997; Garey et al., 2003; Pfaff, 2006; Jing et al., 2009; Lebestky et al., 2009; Agmo, 2011; Van Swinderen and Andretic, 2011; Yokogawa et al., 2012). In addition, relatively little is known about how external and internal inputs interact to set and maintain appropriate levels of arousal. Neuropeptides are attractive candidates to modulate these inputs (Pfaff et al., 2008; Bargmann, 2012), yet systematic and comparative interrogations of neuropeptide function have been constrained by behavioral variability across experimental conditions.

Larval zebrafish are especially useful for studying the molecular and cellular control of arousal, as they possess a conserved yet relatively simple nervous system and display arousal-associated behaviors similar to mammals (Prober et al., 2006; Burgess and Granato, 2007a,b; Wolman and Granato, 2012; Chiu and Prober, 2013). Moreover, their small size facilitates uniformly controlled analyses of behavior across experimental manipulations. The divergence between fish and other vertebrates, separated by 450 million years of evolution, may also be used to infer the ancestral regulation of arousal states (Kumar and Hedges, 1998; Garrison et al., 2012).

Table 1. Locomotor behavior analysis of wild-type larvae

	Day 5	Night 5	Day 6	p values		
				Day 5 vs Night 5	Day 5 vs Day 6	Night 5 vs Day 6
Normal light– dark ($n = 80$)	Light	Dark	Light			
Waking activity (s/waking min)	7.78 ± 0.303	1.41 ± 0.048	4.28 ± 0.168	$<$1.0E-16	2.2E-16	$<$1.0E-16
Rest (min/10 min)	0.19 ± 0.041	1.75 ± 0.131	0.51 ± 0.069	$<$1.0E-16	4.7E-07	1.6E-14
Maximum amplitude/movement (pixels)	9.07 ± 0.098	7.13 ± 0.082	8.01 ± 0.108	$<$1.0E-16	2.3E-11	1.4E-09
Movement duration (s)	0.19 ± 0.002	0.20 ± 0.002	0.17 ± 0.002	6.0E-04	2.7E-09	6.5E-14
Movement frequency (Hz)	0.67 ± 0.024	0.10 ± 0.004	0.37 ± 0.014	$<$1.0E-16	$<$1.0E-16	$<$1.0E-16
Bout duration (s)	1.17 ± 0.056	0.36 ± 0.004	0.89 ± 0.032	$<$1.0E-16	1.8E-05	$<$1.0E-16
Bout frequency (s)	0.26 ± 0.005	0.08 ± 0.003	0.18 ± 0.005	$<$1.0E-16	3.3E-16	$<$1.0E-16
Constant dark ($n = 79$)	Dark	Dark	Dark			
Waking activity (s/waking min)	2.18 ± 0.075	1.29 ± 0.050	1.69 ± 0.051	2.2E-16	2.7E-07	4.7E-08
Rest (min/10 min)	1.65 ± 0.129	3.01 ± 0.160	1.80 ± 0.125	2.7E-09	3.0E-01	9.9E-08
Maximum amplitude/movement (pixels)	9.43 ± 0.209	8.03 ± 0.183	9.29 ± 0.209	1.1E-06	4.9E-01	1.6E-05
Movement duration (s)	0.23 ± 0.003	0.20 ± 0.003	0.21 ± 0.003	1.6E-08	8.2E-03	4.1E-04
Movement frequency (Hz)	0.12 ± 0.005	0.08 ± 0.003	0.11 ± 0.004	1.8E-11	2.4E-01	1.7E-09
Bout duration (s)	0.42 ± 0.010	0.36 ± 0.008	0.46 ± 0.010	6.2E-07	1.9E-03	1.7E-13
Bout frequency (s)	0.09 ± 0.003	0.06 ± 0.003	0.08 ± 0.003	1.1E-10	9.0E-03	1.4E-06
Constant light ($n = 79$)	Light	Light	Light			
Waking activity (s/waking min)	5.85 ± 0.224	2.85 ± 0.125	3.55 ± 0.138	$<$1.0E-16	2.1E-14	7.1E-04
Rest (min/10 min)	0.35 ± 0.094	0.59 ± 0.127	0.39 ± 0.049	1.7E-03	1.2E-05	2.1E-02
Maximum amplitude/movement (pixels)	7.94 ± 0.133	6.67 ± 0.119	7.11 ± 0.120	5.3E-13	1.3E-07	2.0E-03
Movement duration (s)	0.18 ± 0.002	0.16 ± 0.002	0.17 ± 0.002	6.7E-13	3.1E-05	2.3E-05
Movement frequency (Hz)	0.52 ± 0.018	0.28 ± 0.012	0.34 ± 0.013	$<$1.0E-16	6.7E-13	2.4E-03
Bout duration (s)	0.91 ± 0.044	0.50 ± 0.018	0.72 ± 0.031	$<$1.0E-16	3.9E-06	4.1E-14
Bout frequency (s)	0.24 ± 0.007	0.17 ± 0.007	0.17 ± 0.005	1.4E-09	1.8E-11	5.0E-01

Values are mean $\pm$ SEM p values from Kruskall–Wallis one-way ANOVA.

Here we establish quantitative assays of behavioral arousal by decomposing multidimensional and complex locomotor behaviors into simple parameters (Wolman and Granato, 2012). Via genetic expression of seven evolutionarily conserved neuropeptides [adenylate cyclase activating polypeptide 1b (adcyap1b), cocaine-related and amphetamine-related transcript (cart), cholecystokinin (cck), calcitonin gene-related peptide (cgrp), galanin, hypocretin, and nociceptin], we demonstrate that arousal behaviors are behaviorally partitioned: spontaneous locomotor activity can be independent of sensory responsiveness. In addition, we define several novel, unexpected, or conserved functions for neuropeptides. These studies constitute the first quantitative and uniformly controlled comparison of molecular regulators of arousal in vertebrates, partition diverse arousal-associated behaviors, and suggest conserved and novel functions of neuropeptides in regulating arousal.

Materials and Methods

Generation of transgenic fish. Peptides with discrete patterns of expression in the CNS of larval zebrafish were identified by transcriptome profiling and screening via *in situ* hybridization (I. G. Woods and A. F. Schier, unpublished observations). Primers were designed to amplify open reading frames of these peptides via RT-PCR (*adcyap1b*: ATGGCC AGATCTAGTAAAGCG, CTACAAATAAGCAAATCGACGTC; *cart*: ATGACCATGGAGAGTTCCAAAA, TTACAAACACTTCAACAAAA AGTAATTG; *cck*: ATGAGCGCTCTCTCTCCG, TTATGATGAGTATTC ATATTCCTCAGC; *cgrp*: CCCTCTGTTTTTGGGACGACT, AACTGT GGACGTGTGGACTG; *galanin*: ATGCACAGGTGTGTCGGT, TTAGG GTTGACTGATCTCTTCTGATG; *nociceptin*: TGAAGTTCCTGCCTCA TTCC, ATGTGACCCGAGCGACCT). PCR amplicons were cloned into a previously described vector (Prober et al., 2006) and verified by sequencing. This vector was modified to be compatible with the tol2 transposon system (Kawakami, 2004) and to include GATEWAY (Life Technologies) recombination sites downstream of the zebrafish heatshock (*hsp70l*) promoter. Embryos were injected at the one-cell stage and raised to adulthood. Founder adults were identified by screening for ubiquitous expression of the target gene in heatshocked larval progeny via *in situ* hybridization. Several founder adults were identified for each

peptide; stable transgenic lines were derived from the founders that produced the strongest and most widespread expression upon heatshock. Transgenic larvae in the behavioral analyses were distinguished from their wild-type siblings by PCR. Accession numbers for transcripts in this study are as follows: *adcyap1b*, NM_214715; *cart*, BQ480503; *cck*, BC066290; *cgrp*, NM_001002471; *galanin*, EH455016; *hypocretin*, NM_001077392; *nociceptin*, NM_001015044. The transgenic line identifiers for transgenes used in this study are as follows: Tg(hsp70l: adcyap1b)a140, Tg(hsp70l:cart)a137, Tg(hsp70l:cck)a138, Tg(hsp70l:cgrp) a136, Tg(hsp70l:galanin)a135, Tg(hsp70l:hcrt)zf12, Tg(hsp70l:nociceptin)a139.

Analysis of spontaneous locomotor activity. Larvae were raised at 28.5°C on a standard 14/10 h light/dark cycle: lights were turned on at 9 A.M. and off at 11 P.M. Recording of rest/wake activity was performed via infrared Bosch Dinion XF LTC0385 cameras (one-third inch sensors, 752×582-pixel resolution; Bosch) as in Prober et al. (2006). Dishes containing 80 larvae of either sex, each in a separate well, were placed in the recording chamber in the evening of the fourth day postfertilization [dpf, ~102 h postfertilization (hpf)], and were heatshocked at 37°C from 12 noon to 1 P.M. on 5 dpf (~122–123 hpf). Analysis of locomotor activity commenced 1 h postheatshock (~124 hpf) and continued through 6 dpf (~157 hpf).

For the frame-by-frame (15 Hz) comparisons between peptide-overexpressing larvae and their wild-type siblings, the raw locomotor data were parsed via Perl and Matlab scripts. A movement was defined as a pixel displacement between adjacent video frames preceded and followed by a period of inactivity of at least 67 ms (the limit of temporal resolution). A movement bout was defined as a continuous cluster of movements separated by ≦1 s of inactivity. Rest latency was defined as in Prober et al. (2006) as the elapsed time between lights out on 5 dpf (~133 hpf) and the first 1 min period of continuous inactivity. Pairwise comparisons between transgenic larvae and their wild-type siblings (Tables 1–3) were performed via Kruskal–Wallis one-way ANOVA and corrected for multiple comparisons via the Holm–Bonferroni method, while comparisons across genotypes (see Fig. 5) were performed using Tukey's honestly significant difference test.

To generate the statistically typical traces of locomotor activity, behavior was examined in 1 min windows, spaced every 12 s, for each larvae in each experiment. The larva and time period that best matched the aver-

Table 2. Analysis of locomotor activity in peptide-overexpressing larvae

	Day 5			Night 5			Day 6		
Hypocretin	Wild type ($n = 17$)	Hypocretin ($n = 45$)	p values	Wild type	Hypocretin	p values	Wild type	Hypocretin	p values
Waking activity (s/waking min)	5.20 ± 0.534	7.55 ± 0.320	4.21E-04	1.55 ± 0.188	2.02 ± 0.109	2.15E-03	3.30 ± 0.404	5.15 ± 0.320	1.18E-03
Rest (min/10 min)	1.08 ± 0.281	0.50 ± 0.086	8.66E-02	4.61 ± 0.345	1.91 ± 0.250	3.63E-06	1.26 ± 0.335	0.56 ± 0.104	2.21E-02
Maximum amplitude/movement (pixels)	8.43 ± 0.175	9.19 ± 0.135	3.79E-03	6.70 ± 0.216	7.82 ± 0.104	1.11E-11	6.83 ± 0.223	8.04 ± 0.108	3.44E-05
Movement duration (s)	0.17 ± 0.005	0.18 ± 0.002	1.77E-01	0.19 ± 0.000	0.21 ± 0.003	1.04E-06	0.16 ± 0.003	0.17 ± 0.002	2.45E-02
Movement frequency (Hz)	0.67 ± 0.058	0.92 ± 0.034	3.96E-04	0.20 ± 0.025	0.34 ± 0.018	8.02E-12	0.42 ± 0.050	0.64 ± 0.035	5.33E-04
Bout duration (s)	1.76 ± 0.279	2.25 ± 0.187	1.91E-02	0.67 ± 0.103	0.76 ± 0.031	2.59E-09	1.07 ± 0.156	1.47 ± 0.164	2.08E-02
Bout frequency (s)	0.19 ± 0.013	0.22 ± 0.008	8.12E-02	0.11 ± 0.009	0.17 ± 0.006	3.88E-12	0.16 ± 0.012	0.21 ± 0.007	1.40E-03
Rest latency (1 min)	41.05 ± 13.94	125.91 ± 16.01	1.47E-03						
Adcyap1b	Wild type ($n = 40$)	Adcyap1b ($n = 39$)	p values	Wild type	Adcyap1b	p values	Wild type	Adcyap1b	p values
Waking activity (s/waking min)	4.02 ± 0.266	4.69 ± 0.326	1.14E-01	1.35 ± 0.091	1.33 ± 0.089	9.30E-01	3.40 ± 0.204	3.82 ± 0.252	2.76E-01
Rest (min/10 min)	2.13 ± 0.318	1.18 ± 0.246	1.17E-02	3.41 ± 0.268	3.01 ± 0.287	2.66E-01	2.51 ± 0.251	1.71 ± 0.232	1.93E-02
Maximum amplitude/movement (pixels)	7.70 ± 0.141	7.42 ± 0.217	4.39E-01	7.54 ± 0.161	7.37 ± 0.182	7.17E-01	6.92 ± 0.171	6.87 ± 0.200	7.91E-01
Movement duration (s)	0.15 ± 0.002	0.15 ± 0.003	9.22E-01	0.20 ± 0.003	0.20 ± 0.003	5.83E-01	0.14 ± 0.003	0.14 ± 0.003	8.29E-01
Movement frequency (Hz)	0.51 ± 0.039	0.64 ± 0.043	3.21E-02	0.10 ± 0.008	0.11 ± 0.009	5.30E-01	0.43 ± 0.031	0.51 ± 0.030	8.09E-02
Bout duration (s)	1.22 ± 0.091	1.21 ± 0.072	6.73E-01	0.35 ± 0.009	0.36 ± 0.010	4.10E-01	1.39 ± 0.099	1.48 ± 0.097	3.47E-01
Bout frequency (s)	0.20 ± 0.014	0.24 ± 0.013	5.59E-02	0.08 ± 0.006	0.08 ± 0.007	6.10E-01	0.15 ± 0.008	0.16 ± 0.008	1.21E-01
Rest latency (1 min)	24.27 ± 6.31	26.65 ± 5.03	3.88E-01						
Cart	Wild type ($n = 48$)	Cart ($n = 33$)	p values	Wild type	Cart	p values	Wild type	Cart	p values
Waking activity (s/waking minute)	4.08 ± 0.300	4.75 ± 0.488	4.31E-01	0.83 ± 0.042	1.06 ± 0.068	1.50E-02	3.75 ± 0.293	3.64 ± 0.272	9.85E-01
Rest (min/10 min)	1.94 ± 0.342	2.48 ± 0.453	1.91E-01	3.86 ± 0.280	3.05 ± 0.290	8.36E-02	2.59 ± 0.265	2.93 ± 0.328	4.19E-01
Max amplitude/movement (pixels)	7.40 ± 0.249	7.50 ± 0.248	9.69E-01	6.91 ± 0.217	7.01 ± 0.216	9.85E-01	7.01 ± 0.248	6.93 ± 0.262	8.33E-01
Movement duration (s)	0.16 ± 0.004	0.17 ± 0.004	2.82E-01	0.19 ± 0.004	0.20 ± 0.004	2.41E-01	0.15 ± 0.004	0.15 ± 0.004	8.55E-01
Movement frequency (Hz)	0.51 ± 0.034	0.55 ± 0.052	5.97E-01	0.07 ± 0.005	0.09 ± 0.005	6.34E-03	0.43 ± 0.033	0.39 ± 0.029	6.72E-01
Bout duration (s)	1.04 ± 0.070	1.61 ± 0.179	1.25E-02	0.32 ± 0.008	0.36 ± 0.042	7.95E-01	1.29 ± 0.086	1.49 ± 0.138	3.61E-01
Bout frequency (s)	0.22 ± 0.014	0.19 ± 0.017	8.89E-02	0.05 ± 0.004	0.07 ± 0.004	1.13E-02	0.16 ± 0.011	0.14 ± 0.012	2.33E-01
Rest latency (1 min)	16.14 ± 2.69	24.81 ± 3.63	1.12E-02						
Cck	Wild type ($n = 48$)	Cck ($n = 31$)	p values	Wild type	Cck	p values	Wild type	Cck	p values
Waking activity (s/waking min)	3.93 ± 0.22	7.58 ± 0.443	1.10E-09	0.96 ± 0.06	1.02 ± 0.07	2.83E-01	2.08 ± 0.12	2.51 ± 0.19	6.92E-02
Rest (min/10 min)	0.72 ± 0.11	0.87 ± 0.142	2.36E-01	3.69 ± 0.26	4.02 ± 0.40	7.03E-01	1.65 ± 0.20	2.04 ± 0.30	4.13E-01
Maximum amplitude/movement (pixels)	6.45 ± 0.14	6.40 ± 0.204	6.02E-01	6.21 ± 0.13	6.25 ± 0.18	9.36E-01	5.53 ± 0.11	5.39 ± 0.15	3.45E-01
Movement duration (s)	0.16 ± 0.00	0.15 ± 0.003	4.91E-02	0.20 ± 0.00	0.20 ± 0.00	7.56E-01	0.14 ± 0.00	0.14 ± 0.00	2.28E-01
Movement frequency (Hz)	0.55 ± 0.03	1.11 ± 0.051	5.90E-11	0.08 ± 0.01	0.08 ± 0.01	6.81E-01	0.32 ± 0.02	0.39 ± 0.03	9.76E-02
Bout duration (s)	1.12 ± 0.06	4.94 ± 0.501	1.09E-13	0.34 ± 0.01	0.42 ± 0.02	6.18E-04	0.80 ± 0.03	1.15 ± 0.07	2.48E-05
Bout frequency (s)	0.22 ± 0.01	0.13 ± 0.007	6.90E-09	0.06 ± 0.00	0.06 ± 0.01	9.36E-01	0.16 ± 0.01	0.15 ± 0.01	8.37E-01
Rest latency (1 min)	20.48 ± 3.61	21.09 ± 3.89	4.48E-01						
Cgrp	Wild type ($n = 42$)	Cgrp ($n = 37$)	p values	Wild type	Cgrp	p values	Wild type	Cgrp	p values
Waking activity (s/waking min)	4.26 ± 0.26	9.30 ± 0.490	3.10E-11	1.02 ± 0.06	1.23 ± 0.07	2.57E-02	2.56 ± 0.19	3.65 ± 0.34	1.94E-02
Rest (min/10 min)	0.89 ± 0.16	0.09 ± 0.017	3.62E-07	2.98 ± 0.27	1.69 ± 0.25	1.35E-04	1.48 ± 0.21	0.85 ± 0.19	5.52E-04
Maximum amplitude/movement (pixels)	7.20 ± 0.15	7.23 ± 0.166	8.06E-01	6.82 ± 0.13	7.00 ± 0.16	3.46E-01	6.21 ± 0.12	6.00 ± 0.14	7.22E-02
Movement duration (s)	0.16 ± 0.00	0.16 ± 0.002	5.56E-01	0.21 ± 0.00	0.22 ± 0.00	2.23E-01	0.15 ± 0.00	0.14 ± 0.00	5.67E-02
Movement frequency (Hz)	0.55 ± 0.04	1.28 ± 0.058	3.50E-12	0.07 ± 0.01	0.10 ± 0.01	5.27E-03	0.36 ± 0.03	0.55 ± 0.05	2.64E-03
Bout duration (s)	1.15 ± 0.10	5.40 ± 0.759	6.43E-11	0.36 ± 0.01	0.37 ± 0.01	2.50E-01	1.00 ± 0.07	1.47 ± 0.17	1.84E-02
Bout frequency (s)	0.22 ± 0.01	0.18 ± 0.013	3.14E-02	0.06 ± 0.00	0.08 ± 0.01	1.01E-02	0.15 ± 0.01	0.18 ± 0.01	2.38E-02
Rest latency (1 min)	17.84 ± 3.11	42.30 ± 6.53	9.60E-06						
Galanin	Wild type ($n = 23$)	Galanin ($n = 57$)	p values	Wild type	Galanin	p values	Wild type	Galanin	p values
Waking activity (s/waking min)	5.07 ± 0.34	2.52 ± 0.131	3.76E-09	1.64 ± 0.11	1.81 ± 0.09	3.75E-01	4.45 ± 0.41	4.30 ± 0.23	9.11E-01
Rest (min/10 min)	0.96 ± 0.36	3.78 ± 0.199	7.25E-10	2.22 ± 0.30	2.49 ± 0.21	5.17E-01	1.03 ± 0.23	1.01 ± 0.15	9.32E-01
Maximum amplitude/movement (pixels)	7.50 ± 0.18	6.67 ± 0.116	5.62E-04	6.02 ± 0.24	6.40 ± 0.12	1.69E-01	7.84 ± 0.12	7.88 ± 0.11	9.79E-01
Movement duration (s)	0.16 ± 0.00	0.15 ± 0.002	8.78E-03	0.17 ± 0.01	0.18 ± 0.00	9.83E-02	0.16 ± 0.00	0.16 ± 0.00	8.69E-01
Movement frequency (Hz)	0.72 ± 0.05	0.36 ± 0.021	1.25E-08	0.21 ± 0.02	0.20 ± 0.01	7.14E-01	0.58 ± 0.05	0.55 ± 0.03	7.62E-01
Bout duration (s)	1.21 ± 0.10	0.93 ± 0.038	2.97E-03	0.43 ± 0.01	0.44 ± 0.01	9.70E-01	1.33 ± 0.16	1.24 ± 0.07	9.87E-01
Bout frequency (s)	0.25 ± 0.01	0.15 ± 0.006	1.01E-09	0.14 ± 0.01	0.13 ± 0.01	7.78E-01	0.20 ± 0.01	0.20 ± 0.01	8.52E-01
Rest latency (1 min)	27.62 ± 7.03	27.27 ± 6.57	7.50E-02						
Nociceptin	Wild type ($n = 21$)	Nociceptin ($n = 57$)	p values	Wild type	Nociceptin	p values	Wild type	Nociceptin	p values
Waking activity (s/waking min)	4.06 ± 0.38	2.96 ± 0.128	1.30E-02	0.96 ± 0.08	1.00 ± 0.04	5.77E-01	2.78 ± 0.23	2.75 ± 0.13	7.14E-01
Rest (min/10 min)	1.26 ± 0.30	1.84 ± 0.155	1.12E-02	2.65 ± 0.38	3.26 ± 0.20	1.01E-01	1.61 ± 0.30	1.78 ± 0.17	4.30E-01
Maximum amplitude/movement (pixels)	7.40 ± 0.34	8.89 ± 0.175	2.93E-04	6.92 ± 0.26	7.82 ± 0.15	4.14E-03	6.57 ± 0.30	7.65 ± 0.15	1.52E-03
Movement duration (s)	0.15 ± 0.00	0.17 ± 0.003	2.68E-04	0.20 ± 0.01	0.22 ± 0.00	4.32E-02	0.15 ± 0.00	0.16 ± 0.00	4.69E-04
Movement frequency (Hz)	0.55 ± 0.05	0.31 ± 0.016	7.95E-06	0.08 ± 0.01	0.07 ± 0.00	2.26E-01	0.35 ± 0.03	0.29 ± 0.01	4.93E-02
Bout duration (s)	1.21 ± 0.12	0.81 ± 0.029	6.29E-04	0.32 ± 0.01	0.36 ± 0.01	9.73E-03	0.93 ± 0.07	0.88 ± 0.04	6.56E-01
Bout frequency (s)	0.21 ± 0.02	0.16 ± 0.007	8.38E-04	0.07 ± 0.01	0.06 ± 0.00	1.51E-01	0.16 ± 0.01	0.14 ± 0.01	7.79E-01
Rest latency (1 min)	20.25 ± 5.72	13.42 ± 1.46	5.77E-02						

Values are mean ± SEM p values from Kruskall–Wallis one-way ANOVA.

Table 3. Analysis of locomotor activity in transgenic larvae without heatshock

	Day 5		
Hypocretin	Wild type (n = 11)	Hypocretin (n = 19)	p values
Waking activity (s/waking min)	3.54 ± 0.544	5.26 ± 0.543	3.69E-02
Rest (min/10 min)	3.10 ± 0.911	1.23 ± 0.376	7.39E-02
Maximum amplitude/movement (pixels)	11.60 ± 3.322	12.30 ± 1.318	2.20E-01
Movement duration (s)	0.14 ± 0.007	0.14 ± 0.003	4.01E-01
Movement frequency (Hz)	0.41 ± 0.080	0.61 ± 0.062	5.55E-02
Bout duration (s)	1.49 ± 0.239	1.59 ± 0.125	5.61E-01
Bout frequency (s)	0.16 ± 0.035	0.19 ± 0.018	5.19E-01
Rest latency (1 min)	11.07 ± 1.25	15.96 ± 3.28	8.13E-01
Adcyap1b	Wild type (n = 21)	Adcyap1b (n = 26)	p values
Waking activity (s/waking min)	3.28 ± 0.316	2.93 ± 0.239	4.16E-01
Rest (min/10 min)	1.71 ± 0.382	2.71 ± 0.426	2.19E-01
Maximum amplitude/movement (pixels)	9.80 ± 1.584	9.58 ± 1.618	8.47E-01
Movement duration (s)	0.13 ± 0.003	0.13 ± 0.003	6.38E-01
Movement frequency (Hz)	0.41 ± 0.039	0.36 ± 0.032	3.04E-01
Bout duration (s)	1.07 ± 0.113	1.05 ± 0.108	9.83E-01
Bout frequency (s)	0.19 ± 0.019	0.18 ± 0.021	4.94E-01
Rest latency (1 min)	9.15 ± 2.42	8.16 ± 0.98	9.91E-01
Cart	Wild type (n = 20)	Cart (n = 26)	p values
Waking activity (s/waking min)	3.79 ± 0.368	3.38 ± 0.358	3.80E-01
Rest (min/10 min)	1.52 ± 0.323	1.93 ± 0.510	5.54E-01
Maximum amplitude/movement (pixels)	12.15 ± 2.952	11.68 ± 3.197	5.99E-01
Movement duration (s)	0.14 ± 0.004	0.14 ± 0.005	8.26E-01
Movement frequency (Hz)	0.43 ± 0.038	0.38 ± 0.046	4.30E-01
Bout duration (s)	1.03 ± 0.104	0.99 ± 0.077	9.30E-01
Bout frequency (s)	0.19 ± 0.020	0.17 ± 0.020	5.99E-01
Rest latency (1 min)	38.32 ± 14.55	36.64 ± 20.77	7.09E-01
Cck	Wild type (n = 29)	Cck (n = 17)	p values
Waking activity (s/waking min)	4.50 ± 0.362	3.90 ± 0.257	4.19E-01
Rest (min/10 min)	1.26 ± 0.261	2.20 ± 0.467	9.60E-02
Maximum amplitude/movement (pixels)	13.29 ± 2.039	13.22 ± 2.983	9.73E-01
Movement duration (s)	0.15 ± 0.003	0.15 ± 0.005	6.41E-01
Movement frequency (Hz)	0.49 ± 0.041	0.42 ± 0.033	3.57E-01
Bout duration (s)	1.60 ± 0.241	1.57 ± 0.258	9.73E-01
Bout frequency (s)	0.17 ± 0.012	0.16 ± 0.018	6.74E-01
Rest latency (1 min)	10.19 ± 1.65	13.96 ± 3.13	7.97E-02
Cgrp	Wild type (n = 40)	Cgrp (n = 43)	p values
Waking activity (s/waking min)	3.46 ± 0.276	4.12 ± 0.278	5.56E-02
Rest (min/10 min)	2.48 ± 0.403	1.64 ± 0.337	7.54E-02
Maximum amplitude/movement (pixels)	11.00 ± 1.680	12.18 ± 1.596	1.11E-01
Movement duration (s)	0.14 ± 0.003	0.14 ± 0.003	1.96E-01
Movement frequency (Hz)	0.40 ± 0.035	0.48 ± 0.033	3.77E-01
Bout duration (s)	1.32 ± 0.138	1.11 ± 0.077	6.95E-01
Bout frequency (s)	0.18 ± 0.018	0.22 ± 0.016	9.01E-01
Rest latency (1 min)	47.50 ± 11.14	45.03 ± 9.52	8.26E-01
Galanin	Wild type (n = 19)	Galanin (n = 35)	p values
Waking activity (s/waking min)	3.79 ± 0.368	3.38 ± 0.358	3.80E-01
Rest (min/10 min)	1.52 ± 0.323	1.93 ± 0.510	5.54E-01
Maximum amplitude/movement (pixels)	12.15 ± 2.952	11.68 ± 3.197	5.99E-01
Movement duration (s)	0.14 ± 0.004	0.14 ± 0.005	8.26E-01
Movement frequency (Hz)	0.43 ± 0.038	0.38 ± 0.046	4.30E-01
Bout duration (s)	1.03 ± 0.104	0.99 ± 0.077	9.30E-01
Bout frequency (s)	0.19 ± 0.020	0.17 ± 0.020	5.99E-01
Rest latency (1 min)	9.20 ± 0.76	11.66 ± 1.86	5.86E-01
Nociceptin	Wild type (n = 44)	Nociceptin (n = 44)	
Waking activity (s/waking min)	2.97 ± 0.232	2.78 ± 0.207	6.22E-01
Rest (min/10 min)	2.83 ± 0.388	3.69 ± 0.323	4.01E-02
Maximum amplitude/movement (pixels)	9.57 ± 1.344	9.74 ± 1.146	4.53E-01
Movement duration (s)	0.14 ± 0.003	0.14 ± 0.003	1.76E-01

(Table Continues.)

Table 3. Continued

	Day 5		
Nociceptin	Wild type (n = 11)	Hypocretin (n = 19)	p values
Movement frequency (Hz)	0.33 ± 0.031	0.27 ± 0.026	1.56E-01
Bout duration (s)	0.94 ± 0.074	1.23 ± 0.097	1.12E-02
Bout frequency (s)	0.17 ± 0.015	0.12 ± 0.012	1.41E-02
Rest latency (1 min)	6.40 ± 0.44	6.24 ± 0.42	6.89E-01

Values are mean ± SEM p values from Kruskall–Wallis one-way ANOVA. Larvae with the HS-*nociceptin* transgene exhibited decreases in measures of spontaneous locomotion independent of heatshock, but these decreases were not statistically significant after correction for multiple comparisons.

age locomotor parameters across the duration of the experiment were selected for display.

Analysis of sensory responsiveness. On day five of development (~124 hpf), larvae of either sex were distributed into single wells of a 96-well plate (7701-1651; Whatman), allowing simultaneous tracking of each individual. Locomotor activity was monitored via a videotracking system using a camera with 1392 × 1040-pixel resolution running at 15 Hz (Scout sca1400-30fm, Basler Vision Technologies). Stimulus delivery and quantification of larval motion were performed in Matlab. For both the dark-flash and the tap experiments, stimulus ranges were tested upon wild-type larvae, and the apparatus was set to deliver a range of stimuli that reliably elicited a range of responses, from undetectable (no difference from baseline motion) to saturating (no increase of response probability with stimuli of greater strength).

For dark-flash experiments, larvae were heatshocked at 37°C from 9:30 to 10:30 P.M. of 5 dpf (~131.5–132.5 hpf). Beginning at 11 P.M., larvae were subjected to randomized reductions in white light intensity, from a slight dimming to almost complete darkness. Stimuli were delivered by computer-controlled changes in voltage delivered to a custom-built array of 48 white LEDs. These dark flashes lasted for 10 s and occurred at 60 s intervals for 4 h. For tap experiments, larvae were heatshocked at 37°C from 9:30 to 10:30 P.M. of 5 dpf (~131.5–132.5 hpf). Stimuli were delivered by computer-controlled increases in voltage to a solenoid (Allied Electronics 24-I-12D) attached to the apparatus. Beginning at 3:15 A.M. and continuing for 4 h, the dish containing larvae was subjected to automated mechanical taps of randomized intensities. These taps occurred at 30 s intervals. The timing of stimulus delivery (≥30 s between each stimulus) has been shown to be sufficient to prevent behavioral habituation to repetitive stimuli (Burgess and Granato, 2007a). No declines in responsiveness were observed across the duration of the experiments.

For the dark-flash and tap stimuli, a larva was scored as responding if it displaced >10 pixels within a brief time period following the stimulus (3 s for dark flashes, 0.6 s for taps). Responses of each larva were averaged over 40 replicates of each stimulus intensity for dark flashes, and 45 replicates of each stimulus intensity for taps.

For the heat stimulus experiments, larvae were heatshocked from 8 to 9 A.M. on 6 dpf (~142–143 hpf). One hour after the completion of this heatshock (~144 hpf), the response of the larvae to a 5 min pulse of warm (37°C) water was recorded.

Correction for background locomotor activity. To generalize our tests of sensory responsiveness independent of circadian time, we corrected our measurements for differences in basal locomotor activity. The background probability of motion was calculated by determining the number of larvae that displaced >10 pixels within the appropriate time interval after a "sham" stimulus, which was 15 s before each light stimulus or 5 s before each tap stimulus. These background movement data (320 replicates for lights, 540 for taps) were used to calculate an offset value for each larva that estimated its average basal probability of movement any point in time. By subtracting this background probability of motion, the maximum probability of response (i.e., the level at which responses were asymptotic and did not increase with stimuli of greater strength) was lowered. To correct for this decrease and thus to represent the full range of response in an averaged population (0 to maximal probability of response), we divided by (maximum response minus background offset), which restored the maximum response to its original, asymptotic level.

Thus, for each stimulus level, the probability of response at a given stimulus intensity was calculated via the following equation:

$$\text{corrected } P(\text{response}) = \text{observed value}$$

$$\times \frac{[\text{observed value} - \text{background offset}]}{[\text{max observed value} - \text{background offset}]}$$

The maximum response probability was calculated in each experiment for each genotype (transgenic and wild-type siblings) by determining the average number of individuals that responded to the two strongest stimuli over the many replicates of these stimuli.

Distributions for response latency were obtained by measuring the elapsed time between stimulus and response at the two highest stimulus levels. In our latency measurements, we normalized for background differences in basal movement frequency by expressing latency in terms of the following ratio:

$$\text{corrected latency} =$$

$$\frac{\text{Time to first movement observed after a stimulus}}{\text{Time to first movement observed, independent of a stimulus}}$$

Response threshold was calculated by fitting a sigmoid curve to the corrected response data, and determining the stimulus level at which half of the maximum response was reached. Distributions for response threshold were generated by determining the range of threshold values derived from fitting sigmoid curves to 200 bootstrap replicates for each genotype.

For the responses to heat pulse, the average activity (pixels per second) for each genotype was obtained for a 5 min period before stimulus delivery, and was subtracted from the observed values during the analysis window.

Principal components analysis and phenotypic clustering. Data were aggregated for 17 behavioral measures (waking activity, rest bouts, movement amplitude, movement duration, movement frequency, bout duration, bout frequency, rest latency, tap latency, tap response, tap threshold, dark latency, dark response, dark threshold, heat maximum, heat response, heat recovery) and seven peptides (hypocretin, adcyap1b, cart, cck, cgrp, galanin, and nociceptin). Each data point was defined as the ratio of the response of transgenic animals to the response of their wild-type siblings. The data were then log-transformed to ensure that both positive and negative ratiometric values were numerically equivalent, and z-transformed (for each peptide) to place all peptides on a common scale. Phenotypic profiles for the 17 behavioral measures were clustered by similarity in Matlab.

The dataset was then subject to a singular value decomposition to identify the eigenvectors and their corresponding eigenvalues. The generality of the resulting space was evaluated in two ways. First, a jackknife analysis of the data was performed, decomposing a dataset comprising each possible subset of six peptides. Next, to construct a null dataset, a matrix consisting of our dataset was decomposed after it had been randomly shuffled; this analysis was repeated 10,000 times to define the relevant distributions of possible eigenvector values. The eigenvectors and projections from the jackknife analysis were compared with those generated from the full dataset, and against those generated from random data.

Results

Analysis of spontaneous locomotor behaviors

Animals in an increased state of arousal have been proposed to exhibit increased spontaneous locomotor activity and increased sensory responsiveness (Pfaff, 2006). To analyze spontaneous locomotor activity in larval zebrafish, we quantified locomotor behaviors via a videotracking assay (Prober et al., 2006). As previously reported (Prober et al., 2006; Rihel et al., 2010), larval zebrafish after 5 dpf (120 hpf) exhibit robust differences in locomotor behavior between day and night (Fig. 1A). Fish were especially active during the day (Table 1, Fig. 1A); in contrast, periods of rest were increased at night (Table 1).

Although these analyses are suitable for distinguishing overt behavioral differences, they do not completely describe the un-
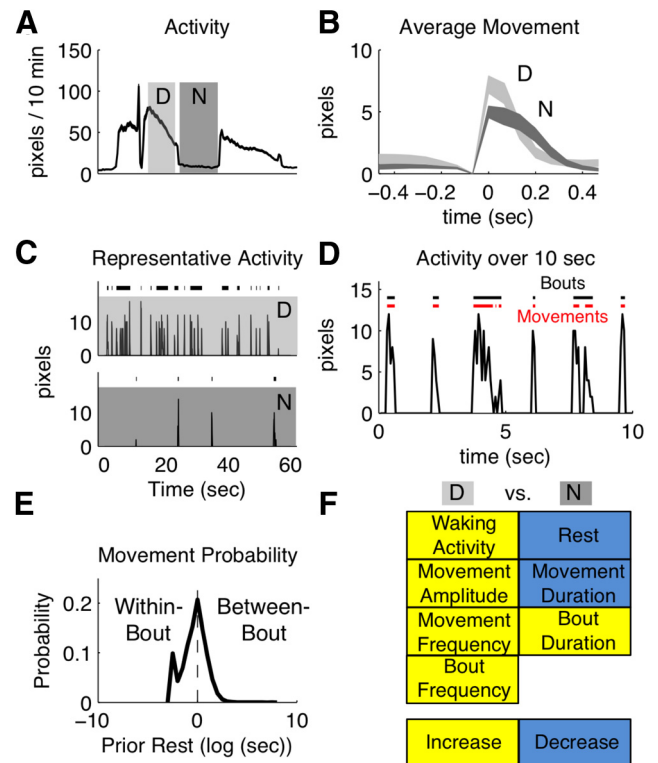

Figure 1. Definitive parameters of spontaneous locomotion in zebrafish larvae. **A**, Mean activity plot for 80 larvae between 5 and 6 dpf (~119–157 hpf). Light and dark boxes represent the period of time during which locomotor activity is analyzed (D, day 5; N, night 5). The sharp increase and decrease in activity immediately before the first box represents the effect of a 1 h heatshock at 37°C. Each data point represents the number of pixels displaced in a 10 min time window, and the width of the line represents ±SEM. **B**, Traces showing average profiles of individual movements, measured by pixel displacement at 15 Hz resolution during day (light shading) and night (dark shading). The extent of shading represents ±SD. Daytime movements are ~25% larger in amplitude ($p < 1 \times 10^{-16}$), and ~5% shorter in duration ($p = 6 \times 10^{-4}$). **C**, Traces showing typical patterns of activity over 60 s for day (light shading) and night (dark shading). Average values were compiled for five parameters of locomotor behavior (movement amplitude, movement duration, movement frequency, bout duration, bout frequency) during each period of analysis. These parameters were used in a search among all larvae to find the best fitting 1 min window of locomotor activity. Movement bouts are indicated by horizontal black lines above each activity trace. Movement frequency was ~7 times higher during the day ($p < 1 \times 10^{-16}$); movement bouts were ~3 times longer ($p < 1 \times 10^{-16}$) and ~3 times more frequent ($p < 1 \times 10^{-16}$) during the day. **D**, Movements are clustered into bouts. The activity of a single larva is shown by measuring pixel displacement sampled at 15 Hz over 10 s. Individual movements (separated by ≤67 ms) are indicated by red lines above the activity trace, while clustered movements (separated by ≤1 s) are grouped into bouts of motion and delineated by black lines above the plot. **E**, The most common interval between movement initiation is 1 s, as shown by comparing probability of movement with time elapsed since the last movement, using data underlying the day 5 locomotion shown in **A**. **F**, Summary of statistical comparisons between day 5 and night 5 locomotor data. Statistically significant increases are shown in yellow, while statistically significant decreases are shown in blue. See Table 1 for *p* values. Significance thresholds at the 0.05 level were corrected for multiple comparisons with the Holm-Bonferroni method.

derlying differences in locomotor activity. For example, they do not answer the following questions: do differences in activity result from changes in the strength or frequency of individual movements, in the organization of individual movements into contiguous bouts, or some combination of these parameters? To address these questions, we sampled locomotor activity at a resolution of 15 Hz (Table 1), allowing us to quantify movement frequency (the number of times a movement was initiated per second), movement duration (the average length of individual movements before returning to rest), and movement amplitude
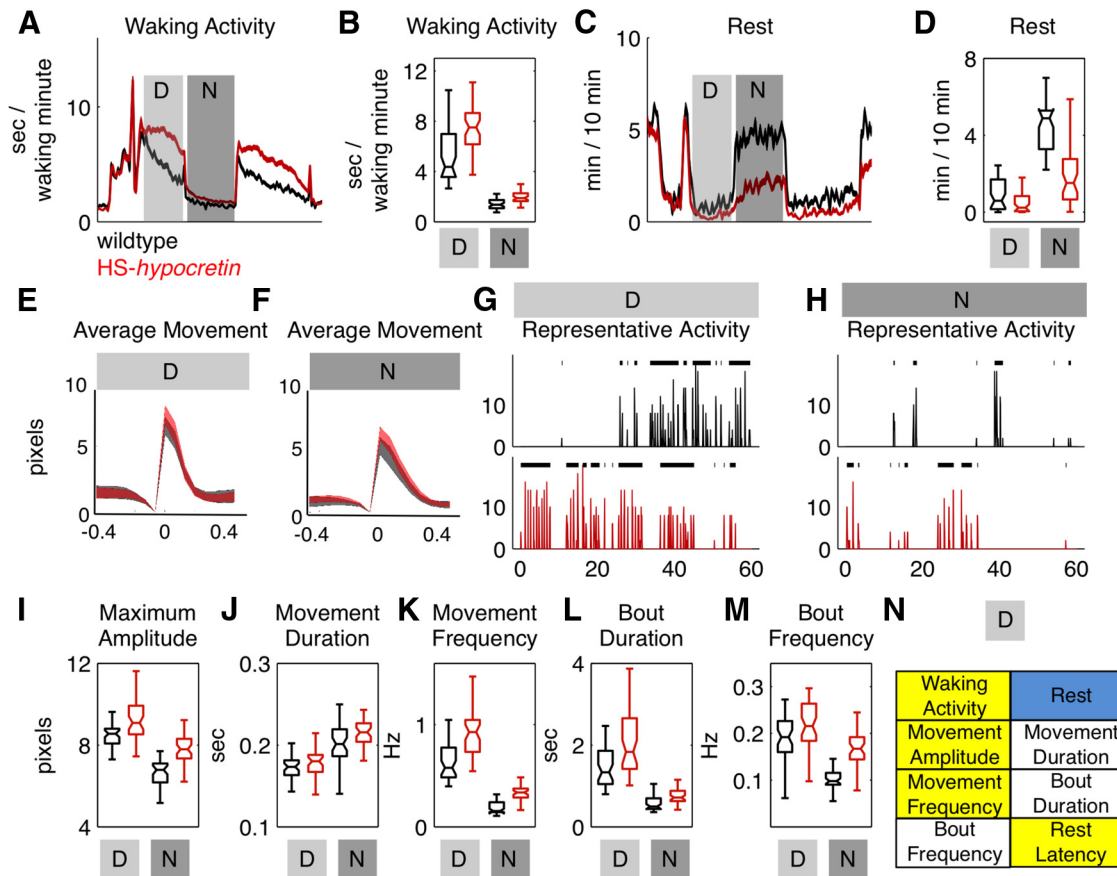
Figure 2. Effects of hypocretin on locomotor activity. **A–D**, Mean waking activity (**A**) and rest bout (**C**) plots and quantitative comparisons (**B**, **D**) for larvae between 5 and 6 dpf. Light and dark shading represents the period of time during which locomotor activity is analyzed (D, day 5; N, night 5). Hypocretin (red) induced striking increases in waking activity (**A**, **B**) and decreases in rest bouts (**C**, **D**), compared with wild-type siblings (black). For the box-and-whisker plots in **C**, **D**, and **I–M**, the analysis timeframe is indicated by light and dark shading as in (**A**), the horizontal lines show the medians, the notches correspond to the 95% confidence intervals around the medians, the open regions delineate the 25th and 75th quartiles, and the lines represent the most extreme data points within the distribution. **E**, **F**, Average single movement traces are shown for day (**E**) and night (**F**), comparing hypocretin-overexpressing larvae (red) with their wild-type siblings (black). **G**, **H**, Traces showing statistically typical patterns of activity over 60 s for day (**G**) and night (**H**). **I–M**, Comparisons of locomotor parameters between hypocretin-overexpressing larvae (red) with their wild-type siblings (black). The most striking differences were in the frequency of movement initiation: hypocretin induced a ∼40% increase in movement frequency during the day ($p = 4 \times 10^{-4}$) and a ∼70% increase at night ($p = 8 \times 10^{-12}$). **N**, Summary of statistical comparisons for locomotor data during the day. Statistically significant increases *hypocretin* larvae are shown in yellow, while statistically significant decreases are shown in blue. See Table 2 for *p* values. Significance thresholds at the 0.05 level were corrected for multiple comparisons with the Holm-Bonferroni method.

(the maximum number of pixels displaced per movement). Traces indicating average activity profiles of wild-type larvae were generated for day and night movement beginning in the afternoon (2 to 11 P.M.) of 5 dpf (∼124–133 hpf), and during the following night (Fig. 1*A*,*B*). In general, daytime movements occurred much more frequently, were of slightly higher amplitude, and were of shorter duration than nighttime movements (Fig. 1*B*,*C*,*F*; Fig. 2*I–K*, wild type; Table 1). Thus, much of the day versus night differences in locomotor activity is driven by the frequency of movement initiation, with only slight changes in the kinematic properties of individual movements.

To distinguish whether the increase in average movement duration at night reflected a genuine circadian influence on locomotion or was an epiphenomenon of nighttime darkness, we repeated the analysis under constant dark and constant light conditions after circadian entrainment (Table 1). Both movement amplitude and movement frequency recapitulated the pattern observed under normal light–dark cycling, with higher activity levels during circadian day. The day–night differences in movement frequency were attenuated in both constant dark and constant light treatments compared with normal light cycling conditions, and we observed a reduction in movement amplitude

during circadian night in both constant dark and constant light conditions. Our analysis of movement duration, however, indicated that longer movements were observed in constant dark and shorter movements in constant light, regardless of circadian time (Table 1). Together, these data suggest that differences in movement frequency and amplitude arise from circadian influences, whereas the difference in movement duration reflects a circadian-independent feature of movement in darkness.

To examine the architectural structure of locomotion, we determined how individual movements are clustered into bouts of activity. Qualitative analysis of activity traces of individual larvae indicated that some movements are isolated, whereas other movements are organized in bouts of movements in rapid sequence (Fig. 1*D*). A plot of elapsed rest versus the probability of motion of individual larvae during day 5 revealed that larvae have the highest probability of motion following a rest interval of ∼1 s (Fig. 1*E*). We thus chose 1 s to delineate the boundary between movement bouts, and thereby defined a movement bout as a continuous group of movements interrupted by ≤1 s of rest. As with the frequency of individual movements, the frequency of movement bout initiation was increased greatly during the day. In addition, bout duration was much greater during day than

night (Fig. 1C; Fig. 2L,M, wild type; Table 1). Thus, once a larva initiated a movement, it tended to remain active for a longer period of time during the daytime compared with night. Together, our high-resolution analysis enabled us to represent complex spontaneous locomotor behaviors by five simple parameters: movement frequency, movement duration, movement amplitude, bout frequency, and bout duration.

To test the potential of our high-resolution analysis in detecting neuropeptide-induced behavioral changes, we focused on hypocretin (also known as orexin). Previous work showed that induction of hypocretin expression in stable transgenic larvae stimulates activity and reduces rest (Prober et al., 2006; Fig. 2A–D; Table 2), but these analyses could not determine which aspect(s) of individual movements were responsible for the differences in overall locomotor activity. On 5 dpf, expression of hypocretin slightly increased the average maximum movement amplitude (Fig. 2E,I,N; Table 2). The increase in movement frequency (Fig. 2G,K,N) was much more pronounced, as were increases in movement bout frequency (Fig. 2G,M,N) and duration (Fig. 2G,L,N). Similar increases for all of these parameters were observed on the following night (Fig. 2I–M; Table 2). Increases in all measured parameters extended similarly through 6 dpf (Table 2). Thus, hypocretin greatly increased the frequency of movement initiation and the length of movement bouts, with minimal effects on the amplitude and duration of individual movements. This analysis highlights the power of our high-resolution assays to uncover differences in spontaneous locomotor activity and to elucidate neuropeptidergic effects upon these behaviors.

Analysis of sensory responsiveness

An animal in an elevated arousal state should exhibit, in addition to increases in spontaneous locomotor activity, increased responsiveness to sensory stimuli of multiple modalities (Pfaff, 2006). To analyze sensory responsiveness, we equipped our videotracking apparatus with computer-controlled delivery of diverse stimuli. This novel paradigm facilitated delivery of changes in light, sound, and temperature in parallel to multiple larvae, and enabled collection of locomotor response data in real time (Fig. 3A–D).

Previous work indicated that sudden onset of darkness induces robust locomotor responses in zebrafish larvae (Prober et al., 2006; Burgess and Granato, 2007b). We found that the magnitude of these responses correlated with the strength of the stimulus (Fig. 3A), where a weak stimulus consisted of a slight dimming of ambient light and a strong stimulus was a transition to almost complete darkness. Varying the strength of such "dark-flash" stimuli produced a sigmoidal response curve, with asymptotes at the basal level of locomotion with weak or no stimuli and the maximal level of response at the strongest stimuli (Fig. 3A,C,E). Similarly, responses to automated taps of various intensities (from inaudible to asymptotic) were collected and analyzed in a manner analogous to the dark flashes (Fig. 3B,C,F). To analyze the response to a thermal stimulus, we briefly exposed the larvae to warm (~37°C) water and assessed changes in locomotor activity (Prober et al., 2008; Fig. 3D,G).

We established nine individual components of responsiveness of larval zebrafish to sensory stimuli. From both the dark-flash and tap analyses, we extracted three parameters of response behaviors: (1) probability (the maximal response observed in terms of percentage of larvae responding; Fig. 3C), (2) threshold (the stimulus strength sufficient to induce a response at half of its maximal value; Fig. 3C), and (3) latency (the time elapsed between a stimulus and the first observed movement). From the
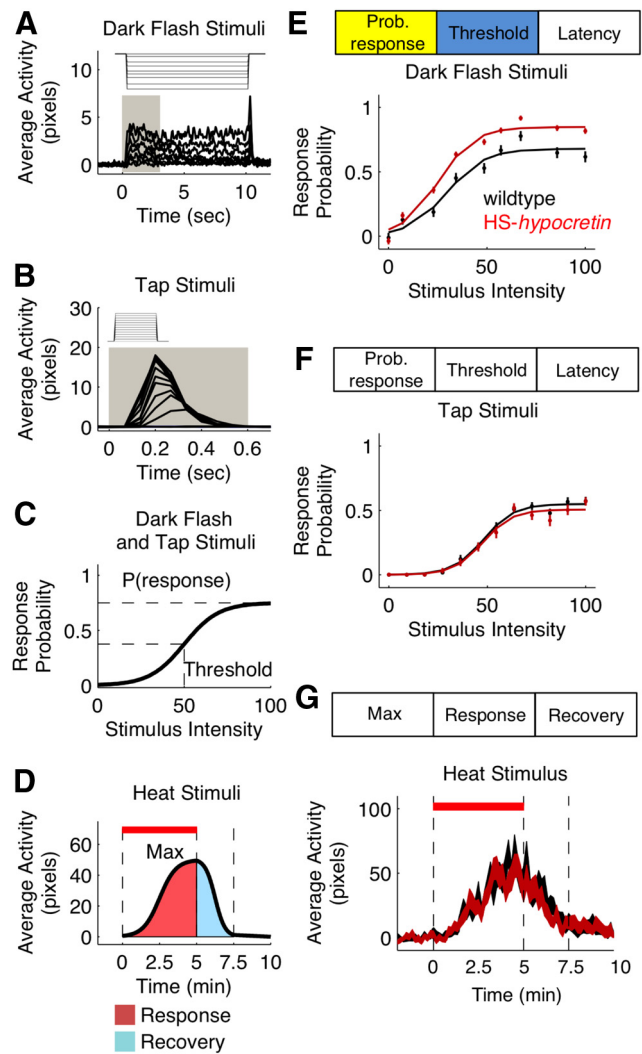

Figure 3. Effects of hypocretin on sensory responsiveness. *A*, *B*, Overview of the dark-flash and tap stimulus paradigms. *A*, *B*, Average locomotor responses of 96 wild-type larvae at 5 dpf [~133–137 hpf for dark flashes (*A*), ~137.25–141.25 hpf for taps (*B*)] to stimuli of varying intensity are shown. *C*, Response curves are generated via analysis of the locomotor data from *A* and *B* within a brief period of time following the stimuli (*A*, *B*, gray boxes; 3 s following dark flashes, and 0.6 s following the taps). Three response parameters are calculated: (1) responsiveness, or the maximum probability of response at the strongest stimuli; (2) threshold, or the stimulus strength at which the half-maximal response is reached; and (3) response latency, or the average time elapsed between the stimulus and initiation for activity. *D*, Overview of the thermal stimulus paradigm. A 5 min heat pulse is delivered to larvae on 6 dpf (~144 hpf). Three behavioral parameters are recorded for each larva: (1) the maximal activity during the heat exposure, measured in pixels displaced per second; (2) the total response, measured in total pixels displaced during the heat exposure; and (3) the recovery, measured in total pixels displaced during the 2 min following heat exposure. *E–G*, Response of hypocretin-overexpressing larvae to sensory stimuli. Statistical comparisons are summarized as in Figures 1 and 2. Compared with their wild-type siblings, hypocretin-overexpressing larvae showed no change in response latency to dark-flash stimuli (*E*), but a striking increase in probability of response ($p = 6.6 \times 10^{-11}$) and a decrease in response threshold ($p = 1.7 \times 10^{-4}$). In contrast, hypocretin-overexpressing larvae showed no changes in response to tap (*F*) or thermal (*G*) stimuli. Width of the line in *G* represents ±SEM. See Table 4 for statistical comparisons. Significance thresholds at the 0.05 level were corrected for multiple comparisons with the Holm-Bonferroni method.

heat-response analyses, we extracted three parameters: (1) maximal magnitude of response, (2) total locomotor activity exhibited over the course of stimulus application, and (3) total activity exhibited during the recovery period as temperature returned to normal (Fig. 3D).
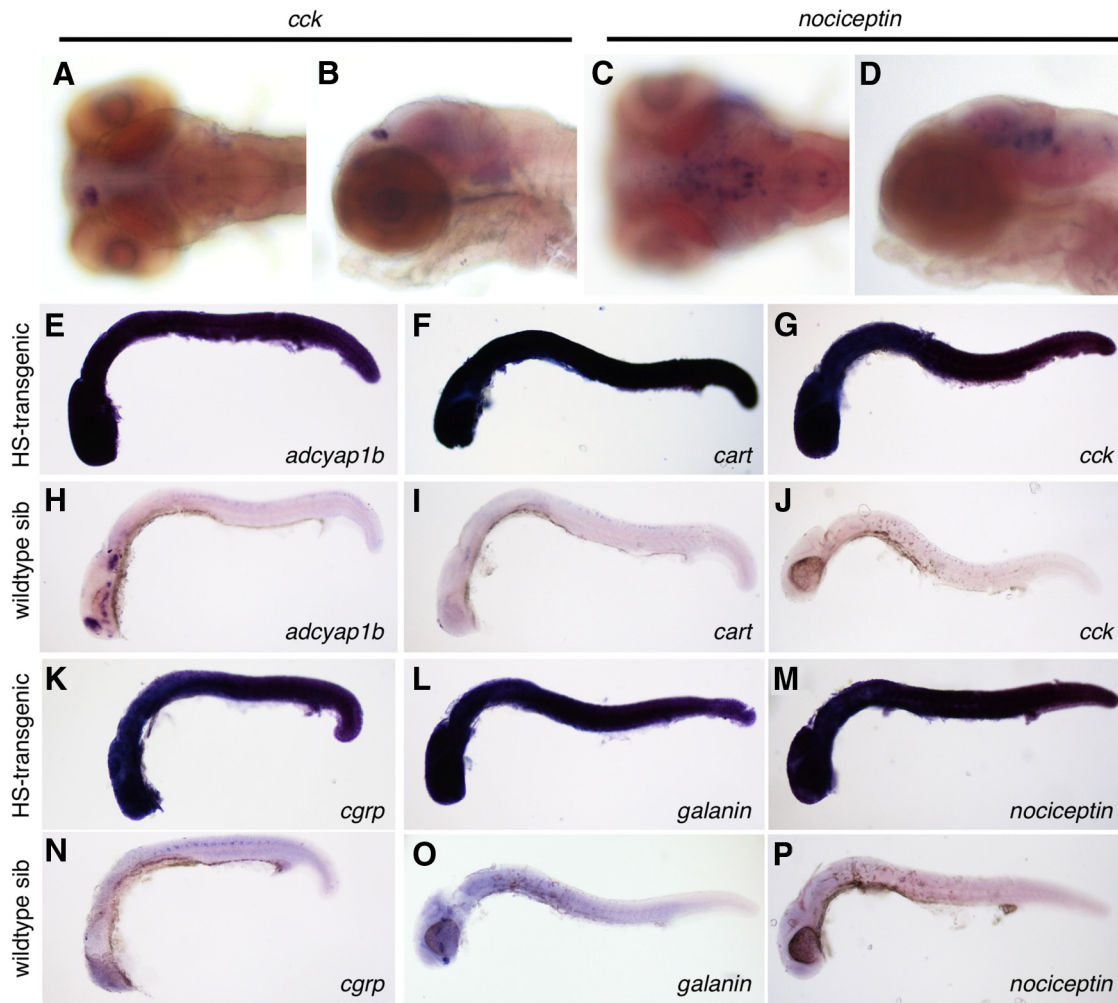
Figure 4. Expression of cck and nociceptin at 5 dpf, and ubiquitous induction of peptide expression by heatshock. ***A–D***, Expression of the indicated transcripts via *in situ* hybridization analysis, viewed dorsally (***A***, ***C***) and laterally (***B***, ***D***) at 5 dpf. Cck (***A***, ***B***) was expressed prominently in the left habenula, while nociceptin (***C***, ***D***) was expressed in clusters of cells localized symmetrically within the brain. ***E–P***, Expression of indicated transcripts in transgenic embryos and their wild-type siblings at 1 dpf. Embryos were heatshocked for 1 h at 37°C, and were fixed 2 h postheatshock for *in situ* hybridization. Upon heatshock, peptide expression was induced ubiquitously in transgenic embryos, but not in their wild-type siblings.

To test the potential of our high-resolution analysis in detecting neuropeptide-induced changes in sensory responsiveness, we exposed heatshock (HS)-*hypocretin* larvae and their wild-type siblings to dark-flash, mechanoacoustic, and thermal stimuli. Because hypocretin induces changes in basal movement frequency (Fig. 2), we corrected our sensory responsiveness measurements for background differences in movement probability (see Materials and Methods). Consequently, in our responsiveness experiments, we report the probability that an observed movement resulted from the stimulus, independent from any differences in basal locomotor activity. Even after these corrections, HS-*hypocretin* larvae exhibited strikingly increased overall responsiveness to the dark-flash stimuli, and a concomitant decrease in the response threshold (Fig. 3E). In contrast, the responsiveness of HS-*hypocretin* larvae to acoustic and thermal stimuli was indistinguishable from that of their wild-type siblings (Fig. 3F, G). Thus, although hypocretin strongly elevated behaviors associated with sensory responsiveness, its effects were surprisingly modality-specific, demonstrating that characteristics of responsiveness to diverse stimuli can clearly be independent in larval zebrafish. This analysis illustrates the power of our assay to uncover subtle differences in behaviors associated with sensory re-

sponsiveness, and to elucidate neuropeptidergic effects on these behaviors.

Systematic comparison of neuropeptidergic regulation of arousal

To determine whether the partitioning of arousal behaviors observed upon hypocretin expression can be extended to other neuropeptides, we created stable transgenic zebrafish in which candidate neuropeptides could be inducibly expressed upon heatshock (Fig. 4). We reasoned that this approach would facilitate systematic dissection of arousal behaviors and suggest the evolutionarily conserved functions of molecular regulators of arousal. Peptides were selected to maximize diversity with respect to peptide family and roles in arousal-related behaviors, and to sample peptides known to be expressed within the CNS of zebrafish larvae within the first 5 dpf (Alt et al., 2006; Blechman et al., 2007; Nishio et al., 2012; Podlasz et al., 2012; Fig. 4A–D). Based on these criteria, we chose peptides suggested from mammalian studies to promote sleep (*galanin*), induce (*cck, cart, adcyap1b*) or alleviate anxiety (*nociceptin*), regulate locomotor activity (*adcyap1b, cgrp*), or modulate stress (*adcyap1b*) for further analyses (Crawley and Corwin, 1994; Jenck et al., 1997;

Köster et al., 1999; Kovács et al., 1999; Hashimoto et al., 2001; Reinscheid and Civelli, 2002; Saper et al., 2005; Gavioli et al., 2007; Rogge et al., 2008; Hammack et al., 2009; Schorscher-Petcu et al., 2009; Vaudry et al., 2009; Rotzinger et al., 2010; Ressler et al., 2011; Sink et al., 2011). We studied the effects of neuropeptide expression on arousal behaviors in zebrafish larvae (Figs. 5–9; Tables 2–5).

We chose a gain-of-function approach for three reasons. First, classic studies of neuropeptide function have established the power of gain-of-function assays to characterize neuropeptidergic activities via injection of purified neuropeptide into the brain and analysis of resulting behaviors (Pedersen and Prange, 1979; Södersten et al., 1983; Clark et al., 1984; Sakurai et al., 1998; Nakazato et al., 2001). In this approach, the neuropeptide is expressed at levels sufficient to occupy and activate most if not all its receptors, resulting in a gain-of-function phenotype independent of the site of neuropeptide expression. Although such approaches cannot distinguish between direct and indirect effects, gain-of-function assays have led to the isolation and characterization of signals that regulate specific behaviors in vertebrates (e.g., NPY, oxytocin, vasopressin, ghrelin, hypocretin/orexin; Pedersen and Prange, 1979; Södersten et al., 1983; Clark et al., 1984; Sakurai et al., 1998; Nakazato et al., 2001). We employ a similar strategy by inducing neuropeptide expression at the genetic level. Second, although gain-of-function approaches cannot establish an essential or endogenous role for a neuropeptide, they result in signaling pathway activation that overcomes the problem of redundancy found in loss-of-function assays. Third, the conditional induction of peptide expression only during the behavioral assays avoids the earlier developmental, physiological, or compensatory effects that often limit loss-of-function approaches. For example, block of nociceptin signaling during embryogenesis leads to defects in placode progenitor formation (Lleras-Forero et al., 2013). Although the conditional induction approach cannot uncover functions caused by distinct patterns of activity (e.g., tonic vs phasic release of a neuropeptide), it avoids the indirect effects caused by long-term loss or gain of neuropeptide expression. Based on this rationale, we studied the effects of conditional neuropeptide misexpression on arousal behaviors in zebrafish larvae (Figs. 5–9; Tables 2–5). In total, we obtained 119 different behavioral measurements over
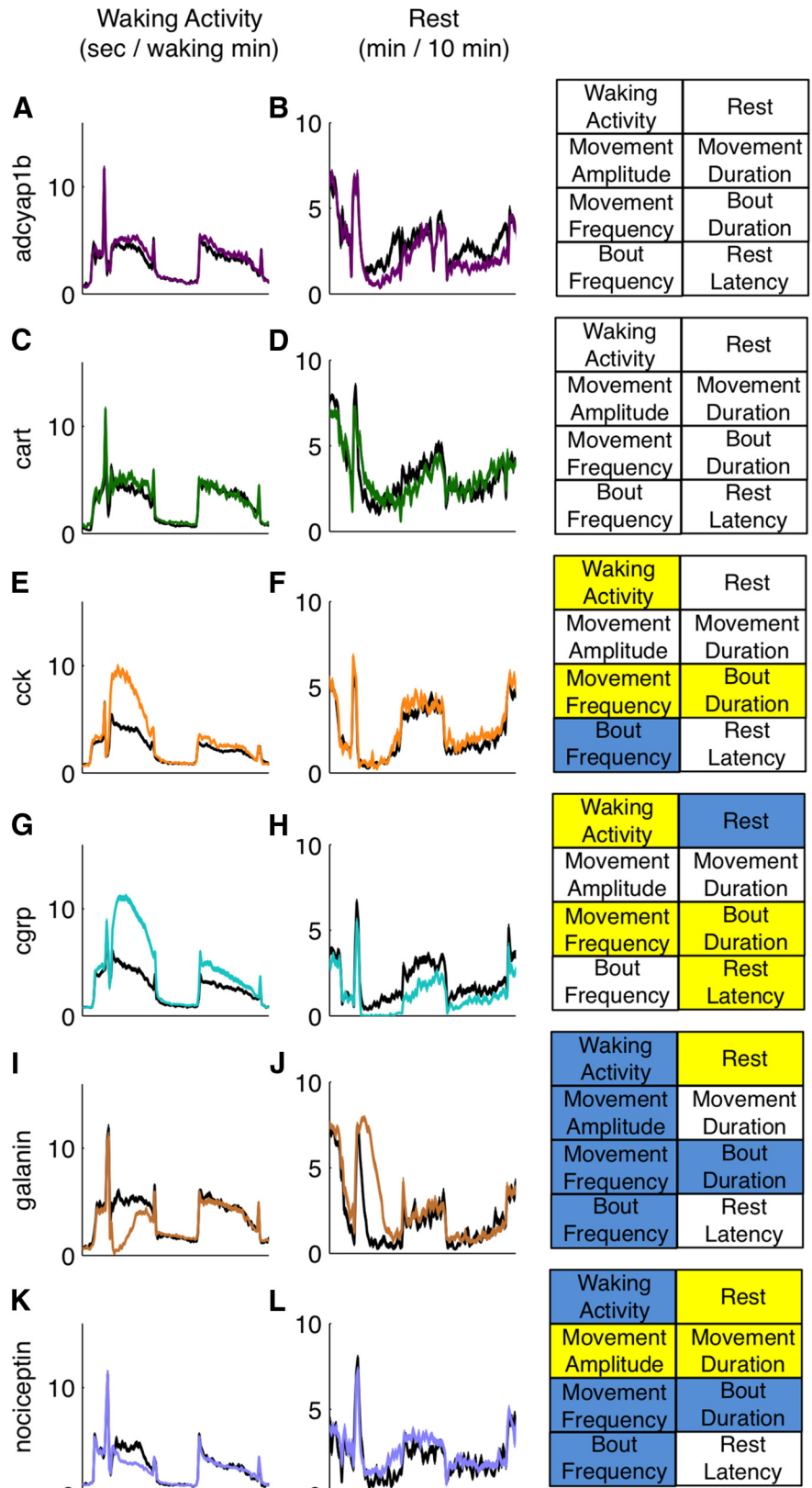

Figure 5. Neuropeptidergic modulation of locomotor activity. Activity and rest plots were generated as in Figure 1 for larvae overexpressing adcyap1b (*A*, *B*, purple), cart (*C*, *D*, green), cck (*E*, *F*, yellow), cgrp (*G*, *H*, aqua), galanin (*I*, *J*, brown), nociceptin (*K*, *L*, blue), and their respective wild-type siblings (black). Shading represents mean ± SEM. Summaries of comparisons between peptide-overexpressing fish and their wild-type siblings are shown to the right of the figure. Statistically significant increases in locomotor behaviors for day 5 depicted in yellow, while statistically significant decreases are shown in blue. See Table 2 for *p* values. Significance thresholds at the 0.05 level were corrected for multiple comparisons with the Holm-Bonferroni method.
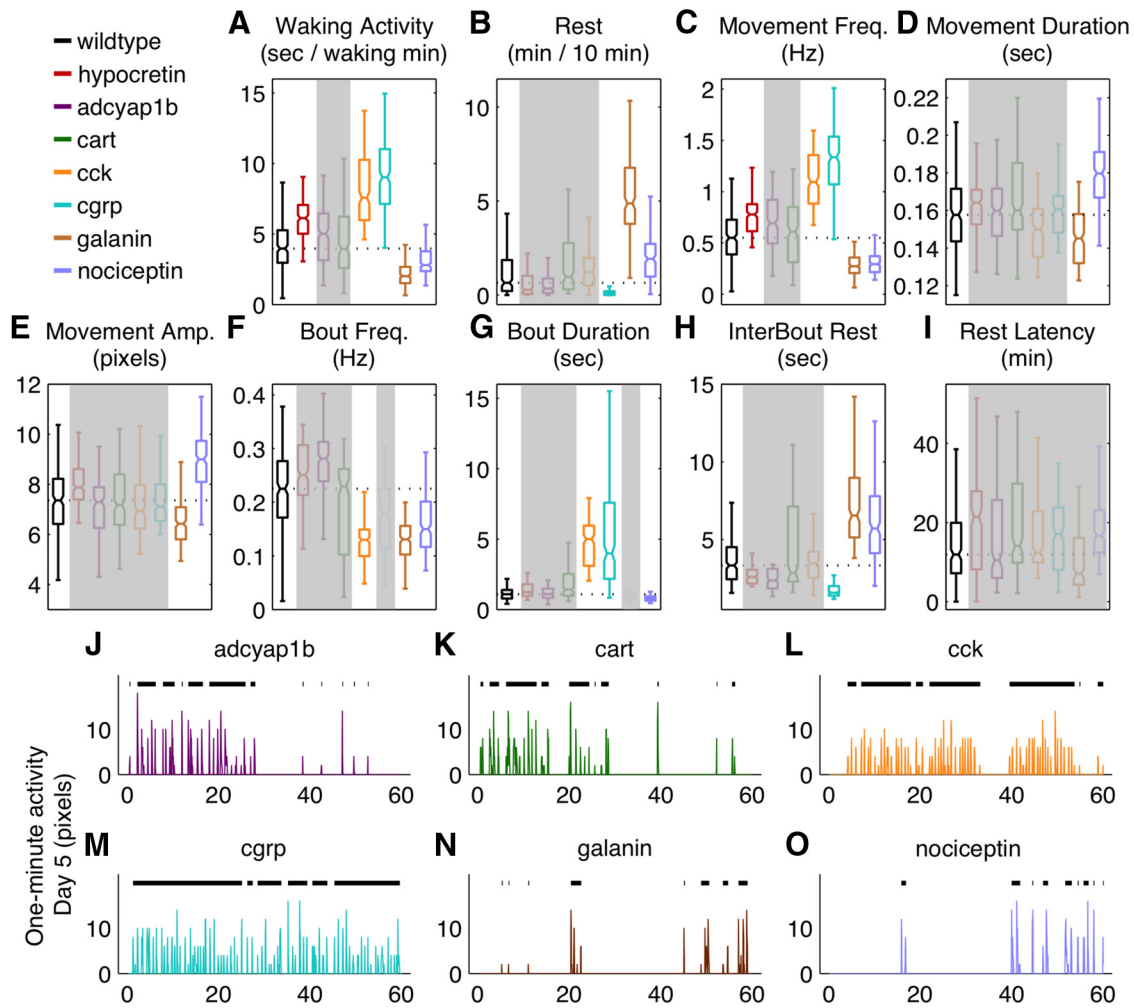
Figure 6. Comparison of peptide-induced locomotor phenotypes on 5 dpf at 15 Hz resolution. Each panel shows box-and-whisker plots as in Figure 2. The median wild-type value for each parameter is shown by the horizontal dashed line. Statistical comparisons were performed by Kruskal–Wallis ANOVA and Tukey's honestly significant difference criteria for multiple comparisons; bold color delineates difference from the wild-type distributions at 99.9% confidence level ($p < 0.001$). **A**, Waking activity, as measured by the total time spent moving in 10 min intervals. **B**, Rest bouts, defined as the number of 1 min spans of continuous inactivity in 10 min intervals. **C**, Movement frequency, where a movement is defined as a pixel displacement preceded and followed by a rest interval at the temporal limit of resolution (i.e., 67 ms). **D**, Movement duration, defined as the average length of pixel displacement per movement, as defined in **C**. **E**, Movement amplitude, defined by the mean maximum amplitude per movement, as defined in **C**. **F**, Bout frequency, where a bout is defined as a cluster of movements separated by ≤1 s of inactivity. **G**, Bout duration, as defined in **F**. **H**, Interbout rest, or the average amount of time elapsed between movement bouts, as defined in **F**. **I**, Rest latency, defined by the average number of minutes elapsed between lights out on 5 dpf and the first 1 min period of inactivity. **J–O**, Statistically typical movement profiles on 5 dpf for the indicated genotypes, based on the 1 min window of data that most closely matches measured values for movement frequency, movement amplitude, movement duration, bout frequency, and bout duration. Movement bouts are delineated by horizontal black lines above each activity trace.

the course of our experiments (7 peptides × 17 measurements). Results of all measurements and peptides are summarized as a heatmap in Figure 9A.

Neuropeptidergic regulation of spontaneous locomotor activity

To test whether neuropeptides influenced spontaneous locomotor activity, we placed transgenic larvae and their wild-type siblings into a videotracking device in the afternoon of 4 dpf (~100 hpf), and induced neuropeptide expression via heatshock at noon on 5 dpf. Locomotor behavior was analyzed through 6 dpf. Peptide expression induced a wide range of phenotypes (Fig. 5; Table 2). For example, cck and cgrp induced increases in waking activity on d5, similar to hypocretin (Fig. 5E,G; Table 2), whereas opposite effects were induced by galanin and nociceptin (Fig. 5I,K; Table 2). In addition, cgrp was similar to hypocretin in that this

peptide induced a reduction in rest (Fig. 5H; Table 2), whereas galanin and nociceptin increased rest (Fig. 5J,L; Table 2).

To determine whether activity and rest are interdependent aspects of locomotor behavior, we compared the rest/wake profiles induced by neuropeptides. Although some neuropeptides (e.g., cgrp and galanin) induced phenotypes in which rest and activity were altered in opposite directions, rest/wake behaviors were not always inversely correlated. For example, cck had no effect on rest bouts (Figs. 5F, 6B; Table 2), in contrast to its strong enhancement of waking activity (Figs. 5E, 6A). In addition, the movement architecture induced by cck was distinct: cck induced longer movement bouts (Fig. 6G) at a lower frequency (Fig. 6F) than other activating peptides (hypocretin and cgrp), yet rest intervals between bouts were equivalent to those of wild-type siblings (Fig. 6H; Table 2). Thus, the comparison of locomotor behaviors across the panel of neuropep-
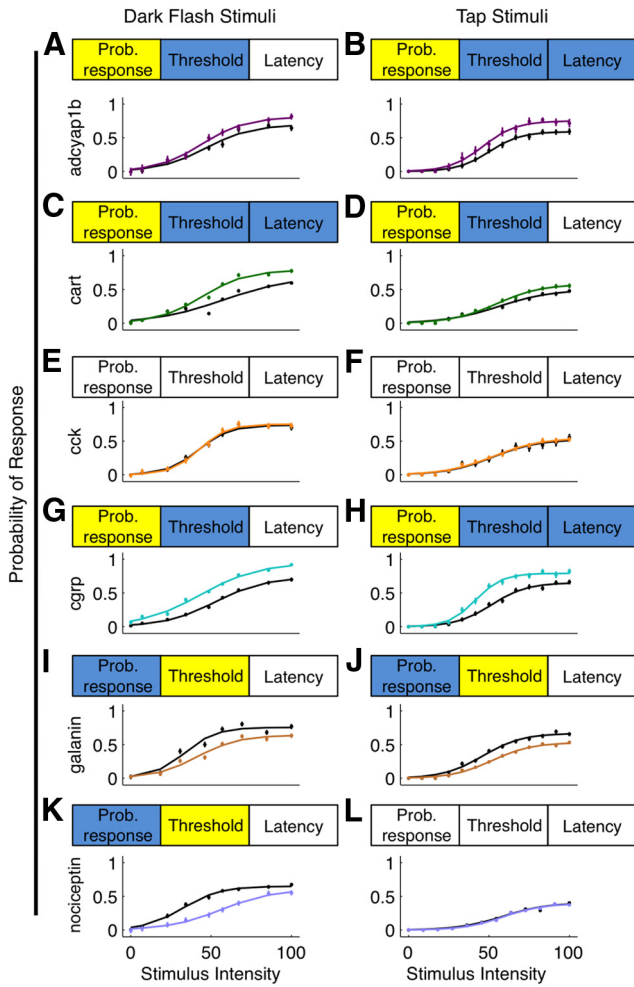
Figure 7. Neuropeptidergic modulation of response to dark-flash and tap stimuli. Stimulus delivery, data acquisition, and response analysis are as in Figure 3. Responses were recorded during circadian night following 5 dpf. Response probability ± SEM is indicated for each stimulus intensity, overlaid with best-fit sigmoid curves. Statistically significant increases are depicted in yellow, while statistically significant decreases are shown in blue. See Table 4 for *p* values. Significance thresholds at the 0.05 level were corrected for multiple comparisons with the Holm-Bonferroni method. *A–H*, Adcyap1b, cart, and cgrp increased probability of response and decreased stimulus threshold for both dark-flash and tap stimuli. In addition, adcyap1b and cgrp decreased latency for response to the tap stimuli (*B*, *H*), while cart decreased latency for response to the dark-flash stimuli (*C*). *E–F*, Responsiveness of cck-expressing larvae was indistinguishable from their wild-type siblings. *I–J*, Galanin decreased probability of response and increased the response threshold for both stimulus modalities. *K*, Nociceptin expression decreased response probability to the dark-flash stimuli, and increased the stimulus threshold. *L*, Responsiveness of nociceptin-expressing larvae to tap stimuli was indistinguishable from that of their wild-type siblings.
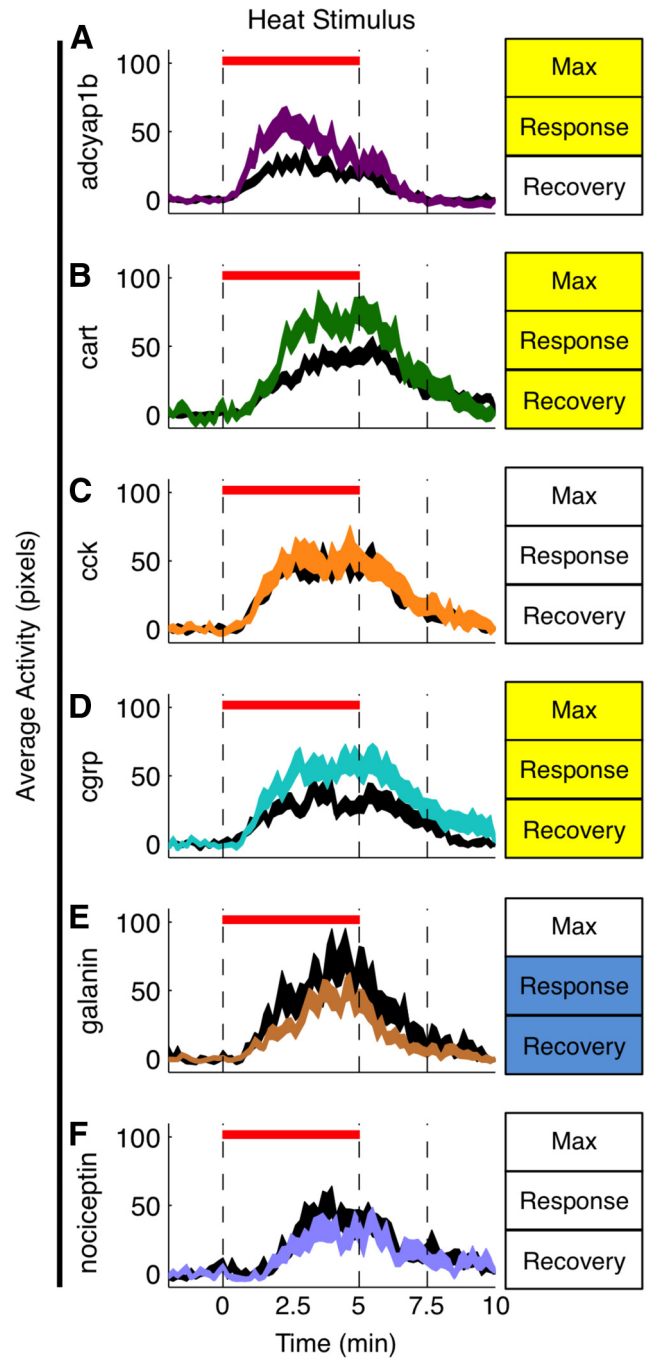

Figure 8. Neuropeptidergic modulation of response to thermal stimuli. Stimulus delivery, data acquisition, and response analysis are as in Figure 3. Responses were recorded during circadian morning on 6 dpf. The width of the plots represents ± SEM. Statistically significant increases are depicted in yellow, while statistically significant decreases are shown in blue. See Table 4 for *p* values. Significance thresholds at the 0.05 level were corrected for multiple comparisons with the Holm-Bonferroni method. *A*, *B–E*, Cart (*B*) and cgrp (*D*) expression induced increases in all measured parameters, whereas adcyap1b (*A*) increased maximum activity and total response, and galanin (*E*) decreased both total response and total recovery. *C*, *F*, Responsiveness of cck-expressing (*C*) and nociceptin-expressing (*F*) larvae to the heat stimulus was indistinguishable from that of their wild-type siblings.

tides indicates that individual components of active and rest states are clearly separable.

To test whether independent behaviors could underlie emergent patterns of locomotor activity, we compared individual parameters of behavior associated with active locomotion. For example, both galanin and nociceptin decreased waking activity during the day (Figs. 5 *I*,*K*, 6*A*), yet nociceptin increased movement amplitude (Fig. 6*E*) and duration (Fig. 6*D*), whereas the opposite effects were observed upon galanin expression (Table 2). To directly visualize and compare these parameters across peptides, we identified periods of locomotion that best exemplified the locomotor attributes quantified for each peptide (Fig. 6*J–O*). Together, this high-resolution analysis revealed that neu-

ropeptides induce wide-ranging and diverse effects on spontaneous locomotor activity.

Partitioning of arousal behaviors

To determine whether spontaneous locomotor activity and sensory responsiveness are behaviorally separable or correlated, we
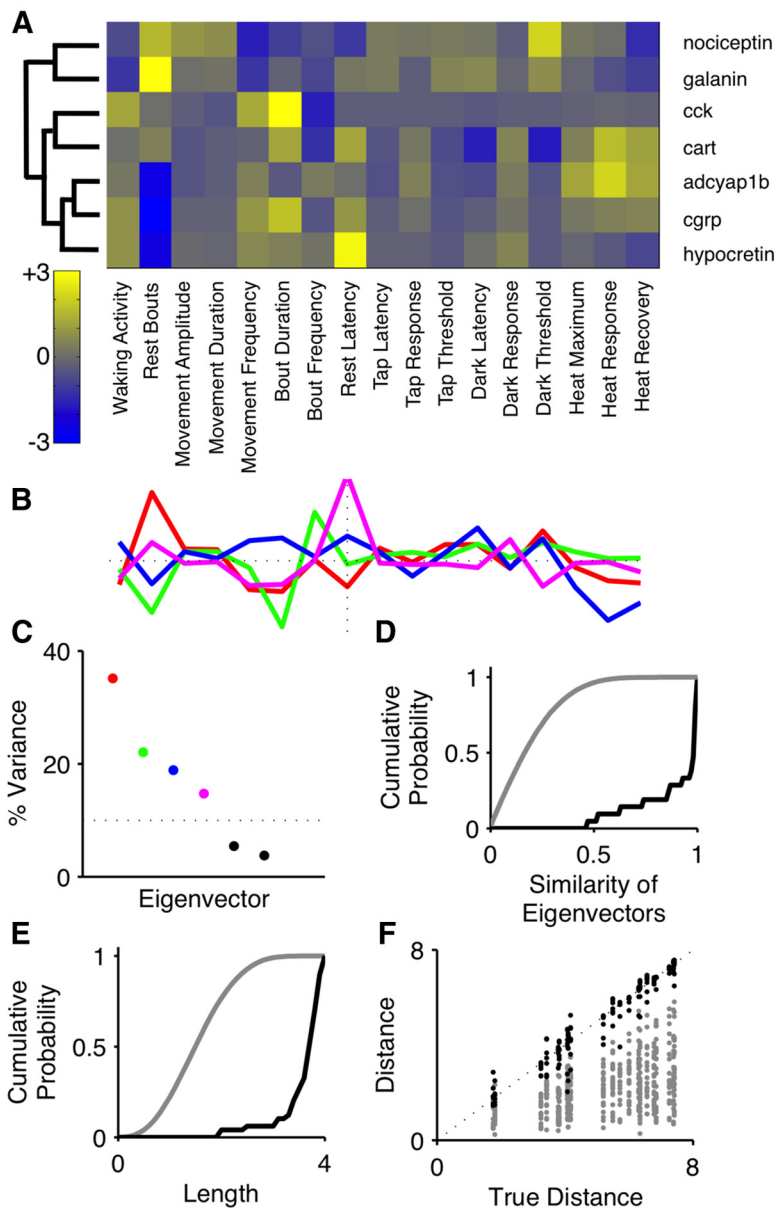
Figure 9. A low-dimensional and general model describing behavioral arousal following neuropeptide expression. To facilitate quantitative comparisons of arousal-related behaviors, we used an unbiased approach to model shared and divergent characteristics of neuropeptide overexpression, and then tested the robustness and generality of this model. ***A***, Clustering of behavioral phenotypes generated a heatmap showing the magnitude (−3–3 log units, blue to yellow) of each of 17 measures against seven neuropeptides. Each square is the *z*-transformed log of the ratio of the response of transgenic animals to the response of their wild-type siblings. Because of normalization of values across all experiments, the blue-yellow color codes do not precisely match those indicated in other figures, which analyzed pairwise differences in phenotype between wild-type and transgenic siblings for an individual neuropeptide. ***B***, The first four eigenvectors generated by the PCA are plotted and colored in red, green, blue, and magenta. ***C***, The variance explained by each eigenvector produced by a PCA using the data in ***A*** is shown in a scree plot; the first four eigenvectors are colored as in ***B***. The dotted line is at 10%. To test how well a novel neuropeptide would perform in our model, we performed PCA on a series of datasets in which each peptide was sequentially omitted, generating a set of new models. These "left-out" models were then compared against null models similarly generated from datasets populated with random permutations of the original dataset. First, we compared the projections of eigenvectors generated by the "left-out" models with those generated by null models. ***D***, The cumulative probability distribution of the projections of eigenvectors derived from randomized datasets is shown in gray, and the distribution of values from the "left-out" model is shown in black. Vector similarity was significantly higher in the "left-out" models. Second, we compared the distance between each peptide and the origin in the three-dimensional space defined by the "left-out" models upon the six remaining peptides (black), to its locus in spaces defined by shuffled data (gray). ***E***, The cumulative probabilities of these distances are shown in gray for shuffled data and in black for the "left-out" models. Vector length was much greater in the "left-out" models than in null models. Third, we compared the interpeptide distance in the "left-out" models with distances in models derived via randomized data. ***F***, The interpeptide distances for all points from the subset of six space versus the seven peptide space are shown in black for the "left-out" models and in gray for shuffled data. The unity line is plotted as a black dotted line. The majority of points lie close to the unity line, suggesting that the spatial arrangement of the peptides remains the same, while peptides in random space tend to cluster more closely.

probed the effects of neuropeptides on the response to changes in illumination, mechanical taps, and heat exposure (Figs. 7, 8; Table 4). For some peptides, behavioral changes were broadly similar between these experiments and the rest–wake analyses. For example, cgrp enhanced both spontaneous activity and sensory responsiveness, while galanin induced the opposite effects. However, we discovered striking exceptions to this trend. While cck was strongly activating in the rest–wake analyses (Figs. 5, 6), it did not affect any parameter of response to optical, mechanical, or thermal stimuli (Figs. 7*E,F*; 8*C*). In contrast, cart and adcyap1b did not alter spontaneous locomotor activity (Figs. 5*A–D*, 6) but enhanced responsiveness to all sensory stimuli examined (Figs. 7*A–D*, 8*A,B*). Thus, neuropeptidergic signaling can uncouple spontaneous locomotor activity and sensory responsiveness.

To test whether peptidergic influences on sensory responsiveness could be modality-specific and partitioned into independent sub-behaviors, we analyzed modality-specific responses. Neuropeptides induced a broad variety of responses to optical, mechanical, and thermal stimuli (Figs. 7, 8; Table 4). Nociceptin did not alter response parameters to taps and heat (Figs. 7*L*, 8*F*; Table 4), but dramatically reduced responsiveness in the dark-flash experiments (Fig. 7*K*; Table 4). Additional differences were observed among the individual parameters (latency, responsiveness, threshold) underlying sensory responsiveness. For example, adcyap1b, cgrp, and cart induced increased responsiveness to all stimuli tested (Figs. 7, 8; Table 4), yet effects on response latency were modality-specific for these peptides. Cgrp and adcyap1b decreased response latency to mechanoacoustic stimuli (Fig. 7*B,H*), while cart induced no differences (Fig. 7*D*; Table 4); in contrast, cart decreased response latency to dark-flash stimuli (Fig. 7*C*), whereas cgrp and adcyap1b expression did not (Fig. 7*A,G*; Table 4).

Thus responsiveness to sensory stimuli can be modality-specific, and behaviors underlying responsiveness to a single modality can vary independently.

Quantitative modeling of behavioral arousal

The diverse behavioral effects of neuropeptides indicated that emergent arousal-associated phenotypes could be partitioned into independent behaviors. To test this idea in an unbiased way, we used a data-driven model. Neuropeptide-

Table 4. Analysis of sensory responsiveness in peptide-overexpressing larvae

	Wild-type siblings	HS-peptide	*p* values
Hypocretin			
Light	n = 22	n = 42	
Responsiveness[a]	0.63 ± 0.03	0.83 ± 0.01	6.60E-11
Threshold	34.94 ± 0.38	32.96 ± 0.27	1.70E-04
Latency	0.51 ± 0.03	0.57 ± 0.03	2.24E-01
Tap	n = 51	n = 43	
Responsiveness[a]	0.57 ± 0.02	0.54 ± 0.03	2.45E-01
Threshold	48.26 ± 0.34	48.38 ± 0.33	6.24E-01
Latency	0.51 ± 0.01	0.51 ± 0.01	9.00E-01
Heat	n = 44	n = 52	
Maximum	167.44 ± 11.592	172.3 ± 8.677	7.34E-01
Response	830.34 ± 82.721	786.21 ± 73.091	6.89E-01
Recovery	496.38 ± 67.384	413.92 ± 53.67	3.35E-01
Adcyap1b			
Light	n = 25	n = 22	
Responsiveness[a]	0.66 ± 0.03	0.79 ± 0.03	1.28E-03
Threshold	47.57 ± 0.68	44.90 ± 0.80	8.20E-03
Latency	0.47 ± 0.02	0.43 ± 0.03	2.24E-01
Tap	n = 18	n = 10	
Responsiveness[a]	0.59 ± 0.03	0.72 ± 0.04	1.52E-02
Threshold	50.78 ± 0.48	47.01 ± 1.04	3.45E-03
Latency	0.71 ± 0.01	0.66 ± 0.01	1.83E-03
Heat	n = 53	n = 42	
Maximum	88.73 ± 6.561	133.98 ± 9.41	1.03E-04
Response	561.88 ± 68.569	1090.6 ± 139.52	4.78E-04
Recovery	170.65 ± 37.142	257.82 ± 71.18	2.53E-01
Cart			
Light	n = 50	n = 46	
Responsiveness[a]	0.57 ± 0.02	0.75 ± 0.02	2.54E-11
Threshold	59.72 ± 1.11	44.60 ± 0.33	<1.00E-16
Latency	0.43 ± 0.01	0.32 ± 0.01	2.39E-08
Tap	n = 55	n = 41	
Responsiveness[a]	0.46 ± 0.02	0.55 ± 0.03	3.36E-03
Threshold	56.78 ± 0.42	54.97 ± 0.35	4.01E-03
Latency	0.44 ± 0.00	0.44 ± 0.00	6.59E-01
Heat	n = 60	n = 35	
Maximum	116.07 ± 6.51	148.18 ± 9.27	4.68E-03
Response	720.05 ± 78.55	1245.60 ± 133.96	4.70E-04
Recovery	574.98 ± 71.78	866.54 ± 110.56	2.32E-02
Cck			
Light	n = 33	n = 31	
Responsiveness[a]	0.71 ± 0.03	0.73 ± 0.03	2.66E-01
Threshold	42.28 ± 0.25	42.66 ± 0.32	4.01E-01
Latency	0.52 ± 0.02	0.51 ± 0.03	4.89E-01
Tap	n = 27	n = 42	
Responsiveness[a]	0.52 ± 0.04	0.52 ± 0.03	9.97E-01
Threshold	53.10 ± 1.05	53.25 ± 0.51	3.38E-01
Latency	0.42 ± 0.01	0.42 ± 0.01	8.92E-01
Heat	n = 47	n = 25	
Maximum	118.25 ± 6.36	123.30 ± 11.21	6.73E-01
Response	959.39 ± 85.11	1067.20 ± 146.29	4.97E-01
Recovery	548.92 ± 71.69	579.46 ± 100.05	8.04E-01
Cgrp			
Light	n = 56	n = 40	
Responsiveness[a]	0.67 ± 0.02	0.88 ± 0.01	3.65E-13
Threshold	52.87 ± 0.28	44.34 ± 0.74	1.13E-13
Latency	0.51 ± 0.01	0.48 ± 0.02	2.32E-01
Tap	n = 27	n = 21	
Responsiveness[a]	0.66 ± 0.02	0.80 ± 0.03	1.34E-04
Threshold	51.89 ± 0.41	42.00 ± 0.37	3.82E-09
Latency	0.44 ± 0.01	0.41 ± 0.00	2.25E-04
Heat	n = 44	n = 49	
Maximum	103.83 ± 8.11	145.41 ± 11.14	3.92E-03
Response	679.98 ± 97.20	1123.80 ± 135.20	1.05E-02
Recovery	436.93 ± 89.09	770.96 ± 112.11	2.39E-02

(*Table Continues.*)

Table 4. Continued

	Wild-type siblings	HS-peptide	*p* values
Galanin			
Light	n = 30	n = 65	
Responsiveness[a]	0.72 ± 0.0249	0.60 ± 0.02	1.02E-02
Threshold	34.2 ± 0.4468	41.305 ± 0.26	2.34E-14
Latency	0.58 ± 0.0481	0.67 ± 0.05	3.53E-01
Tap	n = 41	n = 51	
Responsiveness[a]	0.67 ± 0.02	0.51 ± 0.02	5.87E-08
Threshold	46.40 ± 0.36	52.38 ± 0.25	9.21E-15
Latency	0.43 ± 0.00	0.44 ± 0.00	6.63E-01
Heat	n = 30	n = 65	
Maximum	188.19 ± 15.13	154.87 ± 9.57	5.90E-02
Response	1119.70 ± 137.06	728.35 ± 81.43	1.15E-02
Recovery	615.22 ± 116.48	333.42 ± 64.15	2.43E-02
Nociceptin			
Light	n = 58	n = 37	
Responsiveness[a]	0.66 ± 0.02	0.55 ± 0.03	1.61E-03
Threshold	33.04 ± 0.45	56.85 ± 0.64	2.22E-16
Latency	0.50 ± 0.02	0.48 ± 0.02	4.19E-01
Tap	n = 42	n = 49	
Responsiveness[a]	0.39 ± 0.02	0.38 ± 0.02	5.53E-01
Threshold	59.13 ± 0.52	59.07 ± 0.42	9.43E-01
Latency	0.47 ± 0.01	0.47 ± 0.01	9.62E-01
Heat	n = 40	n = 44	
Maximum	137.73 ± 12.812	134.19 ± 9.8304	8.25E-01
Response	680.57 ± 115.21	627.59 ± 85.381	7.09E-01
Recovery	418.79 ± 80.359	251.65 ± 65.666	1.09E-01

Values are mean ± SEM *p* values from Kruskall–Wallis one-way ANOVA.

[a]Maximum probability of response at the strongest stimuli.

induced phenotypes were summarized in a 7 × 17 grid (Fig. 9*A*), where each row represents a single peptide and each column represents the effect of each peptide on different behavioral parameters, normalized across all experiments. We then built a model of phenotypes by performing a principal components analysis (PCA) on the 7 × 17 grid. Figure 9*C* shows a scree plot of the variance explained by each eigenvector identified in the PCA; the first four (in color) explain >90% of the variance. These eigenvectors (Fig. 9*B*) thus quantitatively define aspects of arousal-associated behavioral phenotypes that covary across our dataset. In the first (red) eigenvector, behaviors associated with increased activity were mostly inversely correlated with behaviors associated with rest. For example, the strong peak at rest bout length was opposite in sign to the peaks associated with movement frequency and latency to rest. In contrast, the other eigenvectors suggest that parameters associated with increased activity could be positively correlated with parameters associated with increased rest. For example, in the second (green) eigenvector, rest bout duration was positively correlated with active bout duration. Similarly, in the third (blue) eigenvector, movement frequency was inversely correlated with responsiveness to sensory stimuli. Thus, activating phenotypes in one assay were sometimes associated with inactivating phenotypes in another, and vice versa. This unbiased quantitative analysis reinforced the conclusion that arousal-related phenotypes could be partitioned into individual behavioral components.

To assess the range of arousal-associated changes in our analyses, we determined how much new information was provided when a novel neuropeptide was added. We ran a series of analyses in which we left out each peptide sequentially, performed a PCA on the remaining peptides, and thereby generated a set of new models, one for each "left-out" peptide. These new models were subjected to three tests to assess how fully our original model

Table 5. Analysis of sensory responsiveness in transgenic larvae without heatshock

	Wild-type siblings	HS-peptide	p values
Hypocretin			
Light	n = 39	n = 35	
Responsiveness[a]	0.70 ± 0.02	0.77 ± 0.02	5.20E-02
Threshold	42.15 ± 0.27	44.31 ± 0.38	3.80E-02
Latency	0.89 ± 0.04	0.86 ± 0.3	8.24E-01
Tap	n = 52	n = 41	
Responsiveness[a]	0.37 ± 0.01	0.35 ± 0.01	3.74E-01
Threshold	42.92 ± 0.38	42.88 ± 0.32	8.16E-01
Latency	0.74 ± 0.01	0.69 ± 0.01	8.77E-02
Heat	n = 11	n = 19	
Maximum	133.01 ± 9.87	134.02 ± 7.29	9.35E-01
Response	948 ± 133.31	661.41 ± 131.13	1.64E-01
Recovery	591.6 ± 101.52	595.53 ± 88.51	9.78E-01
Adcyap1b			
Light	n = 57	n = 38	
Responsiveness[a]	0.74 ± 0.02	0.78 ± 0.02	7.42E-01
Threshold	43.14 ± 0.17	42.50 ± 0.22	2.45E-02
Latency	0.76 ± 0.03	0.79 ± 0.03	3.91E-01
Tap	n = 45	n = 48	
Responsiveness[a]	0.52 ± 0.02	0.51 ± 0.02	7.23E-01
Threshold	35.93 ± 0.06	35.98 ± 0.13	6.01E-01
Latency	0.54 ± 0.01	0.53 ± 0.01	6.28E-01
Heat	n = 57	n = 38	
Maximum	76.67 ± 4.37	89.87 ± 6.14	7.49E-02
Response	364.27 ± 85.43	481.58 ± 113.49	4.03E-01
Recovery	173.23 ± 65.34	161.11 ± 88.68	9.11E-01
Cart			
Light	n = 41	n = 39	
Responsiveness[a]	0.55 ± 0.02	0.60 ± 0.02	2.18E-01
Threshold	44.97 ± 0.64	43.19 ± 0.61	6.67E-02
Latency	0.55 ± 0.02	0.55 ± 0.02	7.60E-01
Tap	n = 42	n = 48	
Responsiveness[a]	0.59 ± 0.02	0.56 ± 0.02	3.37E-01
Threshold	31.41 ± 0.44	31.51 ± 0.27	3.40E-01
Latency	0.85 ± 0.01	0.85 ± 0.01	5.50E-01
Heat	n = 57	n = 36	
Maximum	179.71 ± 9.07	196.67 ± 12.09	2.59E-01
Response	1514.8 ± 102.32	1790.9 ± 173.8	1.47E-01
Recovery	741.51 ± 90.51	786.37 ± 140.48	7.79E-01
Cck			
Light	n = 20	n = 39	
Responsiveness[a]	0.44 ± 0.04	0.44 ± 0.02	9.41E-01
Threshold	49.99 ± 0.64	46.13 ± 0.31	2.32E-02
Latency	0.91 ± 0.06	0.83 ± 0.03	3.70E-01
Tap	n = 27	n = 25	
Responsiveness[a]	0.68 ± 0.03	0.72 ± 0.03	3.13E-01
Threshold	49.33 ± 0.33	48.28 ± 0.46	5.33E-02
Latency	0.68 ± 0.01	0.69 ± 0.01	3.65E-01
Heat	n = 44	n = 40	
Maximum	74.64 ± 5.79	79.68 ± 6.63	5.67E-01
Response	395.19 ± 52.23	459.17 ± 84.13	5.12E-01
Recovery	200.01 ± 45.93	368.35 ± 73.07	5.02E-02
Cgrp			
Light	n = 40	n = 42	
Responsiveness[a]	0.57 ± 0.03	0.58 ± 0.03	5.01E-01
Threshold	43.25 ± 0.27	43.54 ± 0.34	6.76E-01
Latency	0.85 ± 0.04	0.91 ± 0.04	2.73E-01
Tap	n = 39	n = 43	
Responsiveness[a]	0.40 ± 0.02	0.40 ± 0.02	7.57E-01
Threshold	57.32 ± 0.23	57.41 ± 0.25	7.81E-01
Latency	0.47 ± 0.01	0.49 ± 0.01	3.68E-01
Heat	n = 43	n = 40	
Maximum	101.86 ± 6.16	110.92 ± 6.03	2.97E-01
Response	603.57 ± 90.09	585.36 ± 69.53	8.72E-01
Recovery	352.82 ± 72.59	344.08 ± 63.52	9.28E-01

(Table Continues.)

Table 5. Continued

	Wild-type siblings	HS-peptide	p values
Galanin			
Light	n = 46	n = 42	
Responsiveness[a]	0.46 ± 0.03	0.37 ± 0.03	2.11E-02
Threshold	45.44 ± 0.20	45.17 ± 0.33	4.46E-01
Latency	0.55 ± 0.03	0.58 ± 0.03	4.66E-01
Tap	n = 49	n = 47	
Responsiveness[a]	0.62 ± 0.02	0.61 ± 0.02	9.61E-01
Threshold	49.67 ± 0.19	50.44 ± 0.22	9.78E-03
Latency	0.87 ± 0.01	0.87 ± 0.01	7.61E-01
Heat	n = 13	n = 35	
Maximum	131.4 ± 12.32	110.57 ± 6.56	1.19E-01
Response	776.91 ± 117.76	609.45 ± 97.06	3.44E-01
Recovery	513.44 ± 97.57	441.78 ± 67.06	5.70E-01
Nociceptin			
Light	n = 47	n = 48	
Responsiveness[a]	0.72 ± 0.02	0.74 ± 0.02	3.50E-01
Threshold	36.34 ± 0.34	35.69 ± 0.36	2.44E-01
Latency	0.72 ± 0.06	0.66 ± 0.06	3.71E-01
Tap	n = 39	n = 40	
Responsiveness[a]	0.61 ± 0.02	0.61 ± 0.02	9.87E-01
Threshold	48.73 ± 0.23	49.15 ± 0.37	5.57E-01
Latency	0.40 ± 0.01	0.40 ± 0.00	5.35E-01
Heat	n = 44	n = 44	
Maximum	88.98 ± 5.15	95.08 ± 6.40	4.59E-01
Response	501.35 ± 69.98	513.86 ± 59.99	8.92E-01
Recovery	278.61 ± 51.04	338.67 ± 56.13	4.31E-01

Values are mean ± SEM p values from Kruskall–Wallis one-way ANOVA.
[a]Maximum probability of response at the strongest stimuli.

represents the potential range of induced behaviors. First, we took the absolute value of the dot product (a measure of vector similarity, normalized so that 0 indicated no similarity, and 1 indicated a perfect match) between the first four eigenvectors generated in our "left-out" analysis and those derived from the full model. We then compared values with similar values obtained via a "null" model, in which eigenvectors were similarly generated from randomly shuffled data (Fig. 9D). Second, we compared how far each peptide was from the origin in the four-dimensional space defined by PCA upon the six remaining peptides to its locus in a space defined by shuffled data (Fig. 9E). Third, we compared the distance between peptides within our model and a model derived from randomized data (Fig. 9F). All three tests indicated that our dataset encompasses a wide range of possible phenotypes following neuropeptide expression, suggesting that our characterization is general and robust.

Conserved and novel functions of neuropeptides in arousal
Our systematic and parallel analysis allowed us to directly compare the functions of different neuropeptides and their relationships with previously suggested roles in mammalian sleep/wake behaviors, sensory responsiveness, and anxiety.

Spontaneous locomotor activity
Hypocretin
Numerous studies in mammals support a wake-promoting role for hypocretin (de Lecea et al., 2012). These functions are conserved in zebrafish, as described at low-temporal resolution by Prober et al. (2006) and shown at higher resolution in Figure 2.

Cgrp
There are conflicting interpretations of the role of cgrp in mammals. For example, injection of cgrp into the mammalian brain was found to decrease locomotion, whereas inhibition of cgrp

function increases locomotion (Kovács et al., 1999; Schorscher-Petcu et al., 2009). However, other experiments suggest that cgrp increases rearing and grooming movements, and increases anxiety-like behaviors, such as acoustic startle magnitude and time spent in the open arms of an elevated plus maze (Kovács et al., 1999; Sink et al., 2011). Our assays suggest that cgrp may function similarly to hypocretin to increase spontaneous locomotion. Like hypocretin (Prober et al., 2006), expression of cgrp increased locomotor activity and decreased rest (Fig. 6). In addition, cgrp, like hypocretin, induced an insomnia-like phenotype in zebrafish larvae, characterized in part by an increased latency to rest upon night onset (Fig. 6; Table 2; Prober et al., 2006). Our data also suggest that muscle contractions underlying individual movements are unchanged upon cgrp expression, as neither movement amplitude nor duration were increased. Rather, cgrp, similar to hypocretin, increased activity primarily by increasing frequency of movement initiation (Figs. 2G,K, 6).

Cart
Cart peptide has been proposed to promote wakefulness in mammals (Keating et al., 2010). In contrast, cart exerted minimal effects upon sleep/wake behaviors in zebrafish larvae, yet enhanced responsiveness to all sensory stimuli examined (Figs. 5C,D; 6, 7C,D, 8B). Our data therefore suggest that cart may primarily activate sensory responsiveness in larval zebrafish, without affecting spontaneous locomotor activity.

Galanin
Galanin-positive neurons in the ventrolateral preoptic area are active during sleep, send inhibitory projections to structures within the ascending arousal system, and suppress arousal-promoting neurons within the locus ceruleus *in vitro* (Seutin et al., 1989; Pieribone et al., 1995; Sherin et al., 1998, Szymusiak et al., 1998). While these data suggest that galanin may be a sleep-promoting peptide, this function has not been conclusively demonstrated *in vivo*. Our assays uncover striking behavioral changes induced by galanin activity: both spontaneous locomotion and sensory responsiveness were inhibited upon galanin expression. In particular, galanin decreased activity, increased rest, decreased latency to rest at night, and decreased responsiveness to all stimuli examined (Figs. 5I,J, 6, 7I,J, 8E). These sedating effects directly support a role for galanin in promoting rest.

Cck
Acute administration of cck produces panic attacks in humans (de Montigny, 1989). Similarly, activation of the cck pathway increases anxiety-like behaviors in rodents, as measured by changes in locomotor behavior in the elevated plus maze, the light–dark test, and activity in an open field, while blockade of cck receptors attenuates these behaviors (Bowers et al., 2012). In zebrafish larvae, cck induced a unique rest/wake behavioral profile: waking activity was substantially elevated, yet total rest remained unchanged (Figs. 5E,F, 6A,B). Notably, cck induced sustained, contiguous bouts of locomotor activity (Fig. 6G,L). These lengthy movement bouts may represent a "locomotor style" that reflects an anxiety-like state in larval zebrafish (Clark et al., 2011).

Nociceptin
In rodents, intracerebroventricular administration of nociceptin suppresses locomotor activity (Reinscheid et al., 1995; Devine et al., 1996), while disruption of nociceptin signaling enhances motor behaviors (Rizzi et al., 2011). In addition, nociceptin suppresses anxiety-like behaviors in rodents. Specifically, nociceptin

decreases aversion to novel or stressful environments in the several tests that measure locomotor activity preference, including the light–dark box, the elevated plus maze, and exploratory behaviors within a novel environment (Jenck et al., 1997). Similarly, loss-of-function of nociceptin or its receptor induces changes in locomotor activity indicative of an anxiety-like state (Köster et al., 1999; Reinscheid and Civelli, 2002; Gavioli et al., 2007; Rizzi et al., 2011). In our assays, nociceptin reduced waking activity and increased rest (Figs. 5K,L, 6A,B). In addition, movement bout lengths induced by nociceptin expression were the shortest of all peptides tested (Figs. 6G,O, 9A). Thus our data suggest that nociceptin suppresses spontaneous locomotion, and that this function is conserved from fish to mammals.

Sensory responsiveness
Hypocretin
There have been conflicting reports regarding a role for hypocretin in modulating sensory responsiveness in mammals. For example, it has been suggested that administration of hypocretin to the brain can reduce responsiveness to acoustic stimuli (Singareddy et al., 2006), or have no effect (Jones et al., 2001). In contrast, another study suggested that hypocretin signaling enhances sensitivity to these stimuli: pharmacological inhibition of hypocretin signaling decreased responsiveness to acoustic bursts (Steiner et al., 2012). To our knowledge, a role for hypocretin in modulating responsiveness to visual startle or acute changes in illumination has not been previously explored. Here, expression of hypocretin increased responsiveness to dark-flash stimuli (Fig. 3E), suggesting an additional role for hypocretin apart from its well characterized functions in stabilizing wakefulness (Sakurai, 2007; Carter et al., 2013). In contrast, responses to thermal and mechanical/acoustic stimuli were unchanged upon hypocretin expression (Fig. 3F,G). Our assays therefore suggest hypocretin has modality-specific functions in sensory responsiveness, and specifically enhances responsiveness to changes in illumination.

Nociceptin
In mice, pharmacological activation of nociceptin signaling disrupts sensorimotor gating mediated by visual stimuli, but not by acoustic stimuli (Ces et al., 2012), suggesting that this peptide may independently modulate circuits underlying response to different sensory modalities. In contrast, intracerebroventricular injection of nociceptin in mice does not alter responsiveness to thermal stimuli, as assayed by reaction latency in a hot-plate test (Reinscheid et al., 1995). Our assays suggest that nociceptin function is similar between zebrafish and mammals. In particular, nociceptin attenuated responses to changes in illumination (Fig. 7K) without affecting sensitivity to mechanical (Fig. 7L) or thermal (Fig. 8F) stimuli.

Cgrp
Several studies have suggested mechanosensory functions for cgrp. For example, cgrp is expressed in hair cells both in vertebrate ears (Takeda et al., 1987) and in the amphibian lateral line organ (Adams et al., 1987). In addition, infusion of cgrp into the brain has been shown to potentiate the acoustic startle response in rats (Sink et al., 2011). In contrast, intracerebroventricular administration of cgrp decreases responsiveness to thermal stimuli in rats, but only at high doses (Pecile et al., 1987), while effects of cgrp upon visual startle or other responses to rapid changes in illumination are unknown. Our assays suggest that a mechanosensory function may be conserved in fish. In particular, cgrp increased overall levels of response to all stimulus modalities

tested (Figs. 7G,H, 8D), but specifically decreased the reaction time to tap stimuli (Fig. 7H).

Adcyap1b

Infusion of adcyap1 into the rat brain enhances acoustic startle behaviors (Hammack et al., 2009). Interestingly, the sensory phenotypes induced by adcyap1b in larval zebrafish were similar to those induced by cgrp, suggesting conservation of a mechanosensory function. Roles for mammalian adcyap1b in modulating responsiveness to stimuli of other modalities are less clear. Injection of this peptide into peripheral tissues in rat enhances injury-sensitized thermal responsiveness without altering baseline responses to thermal stimuli (Sándor et al., 2009), while a role for adcyap1b in modulating responses to acute changes in illumination has not been described. In our assays, adcyap1b enhanced responsiveness to both thermal stimuli and changes in illumination.

Cart

In mouse, cart is a specific marker of ON–OFF direction-selective retinal ganglion cells, which respond to light onset and termination (Kay et al., 2011), suggesting that cart may function in detecting changes in illumination. Our assays provide behavioral support for this hypothesis. In particular, response latency to dark-flash stimuli was specifically decreased upon cart expression, suggesting a modality-specific role for this peptide (Fig. 7C). Effects of mammalian cart upon stimuli of other modalities are less clear. Though intracerebroventricular administration of cart in mice can decrease response to acoustic startle and thermal stimuli (Bannon et al., 2001; Damaj et al., 2003), the dose of cart used in these studies approaches levels that impair locomotor function (Damaj et al., 2003). In larval zebrafish, expression of cart enhanced responsiveness to stimuli of all modalities tested (Figs. 7C,D, 8B).

Discussion

Our study makes four contributions to the study of arousal: we develop high-throughput and sensitive assays for arousal in zebrafish larvae, provide the first parallel and thus directly comparable analysis of neuropeptidergic modulation of arousal in vertebrates, demonstrate that spontaneous activity and sensory responsiveness are separable aspects of arousal state, and reveal novel, unexpected, or conserved functions for neuropeptides in arousal.

Arousal behaviors

We employ two behavioral indicators of arousal: spontaneous locomotion and sensory responsiveness. Our analysis overcomes limitations of previous high-throughput but low-temporal resolution studies (Prober et al., 2006; Rihel et al., 2010). Notably, the improved temporal resolution enables analysis of individual movements and delineates quantitative characteristics (movement duration, frequency, amplitude, density) that generate broad differences in locomotor activity. We similarly dissect sensory responsiveness by quantifying stimulus threshold, response probability, and latency of response to various stimuli. We thus elucidate discrete parameters underlying complex behaviors, thereby highlighting subtle behavioral differences and facilitating comparisons across experiments.

Clustering of behavioral phenotypes highlighted commonalities and differences between different neuropeptides. For example, we identified a peptide (cgrp) that induced locomotor behaviors similar to those induced by hypocretin, peptides (cck and nociceptin) that induced unique profiles of locomotor activity, peptides that specifically influenced locomotor activity (cck) versus sensory responsiveness (cart and adcyap1b), and peptides that induced modality-specific changes in sensory responsiveness (hypocretin, adcyap1b, cart, cgrp, nociceptin). Quantitative modeling indicated that we explored much of the potential range of arousal behaviors in larval zebrafish. Behavioral clustering is a powerful tool to link experimental manipulations with relevant biochemical and neuronal pathways (Rihel et al., 2010). The work presented here thus enables systematic comparisons of arousal phenotypes induced by genetic and pharmacological manipulations, and will facilitate elucidation of shared biochemical or neuroanatomical functions underlying arousal.

Partitioning of arousal

The operational definition of arousal (Pfaff, 2006)—that an aroused animal moves more and is more responsive to stimuli—has stimulated debate regarding the unitary versus partitioned nature of arousal (Jing et al., 2009; Lebestky et al., 2009; Van Swinderen and Andretic, 2011). Here, neuropeptide expression clearly uncouples spontaneous locomotor activity from sensory responsiveness, and further partitions each into independent behavioral components. Thus our experiments support the hypothesis that arousal is behaviorally separable.

One way to partition behaviors is via differential activity of distinct neuronal circuits. For example, dopaminergic influences upon locomotor activity and sensory responsiveness in *Drosophila* are governed by distinct circuitry. Tissue-specific functional rescue in dopamine receptor mutants indicates that circuits that mediate startle responses are distinct from those that regulate rest–wake behaviors (Lebestky et al., 2009). Similarly, the serotonergic dorsal raphe nucleus (DRN) in larval zebrafish increases visual responsiveness, but not locomotor activity, after an arousing stimulus (Yokogawa et al., 2012). Thus distinct arousal-regulating systems may regulate distinct arousal-related behaviors, and the broad range of behaviors observed here may reflect differential neuropeptidergic activity within individual arousal-generating systems.

Our results also indicate that these systems may be coordinately regulated. For example, cgrp enhanced both spontaneous locomotion and sensory responsiveness, while galanin exerted the opposite effects. Similarly, cart and adcyap1b increased responsiveness to stimuli of all modalities tested. These results suggest that behavioral components of arousal can be both uniformly controlled and independently regulated.

Neuropeptide functions in arousal

Our systematic analysis of arousal phenotypes allows us to suggest functions for neuropeptides via several approaches: we identify conserved and potentially ancestral arousal-related functions, test functions suggested by mammalian studies, propose novel functions for neuropeptides in regulating arousal behaviors, and identify potential antagonistic or cooperative interactions among neuropeptides.

Conserved functions

In mammals, hypocretin neurons innervate several arousal-promoting centers (Carter et al., 2013), and optogenetic activation of these neurons promotes wakefulness (Adamantidis et al., 2007; Carter et al., 2010). Our results strengthen the previous conclusion (Prober et al., 2006) that these functions are conserved. Specifically, hypocretin increased activity and decreased rest, primarily by increasing frequency of movements.

In mammals, nociceptin suppresses locomotion (Reinscheid et al., 1995; Devine et al., 1996; Rizzi et al., 2011), and is associated

with visual sensorimotor gating (Ces et al., 2012). Similarly, nociceptin reduced spontaneous activity and decreased responsiveness to dark-flash stimuli in larval zebrafish.

Mechanosensory functions have been proposed for both adcyap1 and cgrp in mammals (Adams et al., 1987; Takeda et al., 1987; Hammack et al., 2009; Sink et al., 2011). Both of these peptides specifically decreased response latency to tap stimuli in our assays, suggesting that these mechanosensory functions may be conserved.

Neuropeptides that elevated arousal-related behaviors in our assays (adcyap1b, cgrp, cart, cck, and hypocretin) have each been associated with anxiety or stress in vertebrates (Kask et al., 2000; Hashimoto et al., 2001; Chaki et al., 2003; Hammack et al., 2009; Ressler et al., 2011; Sink et al., 2011; Johnson et al., 2012). Inappropriately elevated arousal is associated with disorders of anxiety, and is a defining feature of stress (Pfaff et al., 2007; de Lecea et al., 2012). Cck and nociceptin are among the best-studied examples of neuropeptide function in anxiety: Cck induces anxiety behaviors in mammals (Bowers et al., 2012), while nociceptin reduces these behaviors (Jenck et al., 1997). Interestingly, these peptides induced striking and opposite changes to locomotor bout length in larval zebrafish. In particular, cck expression caused extended bouts of locomotion, whereas bout lengths induced by nociceptin were the shortest of all peptides examined. Locomotor bout length may correlate with arousal state stability, and deviation from normal ranges could reflect defects in consolidation or maintenance of arousal. Extended bout lengths could be indicative of inappropriately stabilized arousal, and may reflect an anxiety-like state.

Proposed functions

Galanin is proposed to be a sleep-promoting peptide (Saper et al., 2005), but its *in vivo* behavioral effects have not been elucidated. We find that galanin decreased both spontaneous locomotion and sensory responsiveness, supporting a generally sedating role for galanin.

Injection of cart into the rat brain has been reported to increase wakefulness (Keating et al., 2010). Cart can also induce anxiety-like behaviors (Kask et al., 2000; Chaki et al., 2003); therefore, the increased wakefulness upon cart administration might have resulted from stressful stimuli during handling and injection (Keating et al., 2010). Our study suggests that cart primarily enhances sensory responsiveness: cart expression increased responsiveness to all sensory stimuli tested, but did not affect spontaneous locomotor activity.

Novel functions

Though cgrp promotes anxiety-like behaviors in rodents (Sink et al., 2011), an explicit function in arousal has not been previously described. Expression analysis suggests that cgrp interacts with multiple arousal-generating centers. For example, both cgrp and a cgrp receptor component are localized to the locus ceruleus and the DRN of rat (Ma et al., 2003). In our assays, cgrp induced locomotor behaviors strikingly similar to those induced by hypocretin; in addition, and in contrast to hypocretin, cgrp elevated responsiveness to all sensory stimuli examined. Therefore cgrp may function similarly to hypocretin in elevating activity, but may have additional roles in modulating sensory responsiveness.

While nociceptin induced modality-specific effects upon responsiveness to changes in illumination (Fig. 7K), consistent with its proposed role in mediating visual sensorimotor gating (Ces et al., 2012), functions for additional neuropeptides in mediating response to acute changes in illumination have not been

described. In our assays, galanin decreased responsiveness to dark-flash stimuli, while adcyap1b, cart, hypocretin, and cgrp increased responsiveness. Furthermore, both hypocretin and cart exerted modality-specific effects: cart specifically decreased response latency to dark-flash stimuli, while hypocretin specifically increased responsiveness to these stimuli. Because serotonergic projections from the DRN to the optic tectum specifically modulate sensitivity to optical stimuli in larval zebrafish (Yokogawa et al., 2012), it may be interesting to determine whether the neuropeptides that alter responsiveness to the dark-flash stimuli may interact with this DRN-tectal circuit.

Antagonistic and shared functions

Similar or opposing behavioral changes induced by neuropeptides may indicate shared sites of activity or mechanisms of function. For example, cgrp and hypocretin may similarly act upon arousal-generating centers to promote wakefulness. In contrast, cgrp and galanin induced opposite behavioral phenotypes, and might therefore exert antagonizing effects upon common targets. Similarly, cck and nociceptin induced opposite changes in locomotor bout length, potentially reflecting antagonistic roles for these peptides in modulating anxiety-like behaviors.

Our study lays the foundation for a large-scale analysis of neuropeptidergic regulation of arousal and raises the question how the diverse behavioral effects induced by neuropeptides are caused at the circuit level (Schier, 2013). Precise manipulations of peptide function at the genetic or cellular level will be needed to test where arousal is modulated, e.g., by sensitization peripherally and/or centrally. *In vivo* interrogation of neuronal activity will be required to determine whether arousal-generating systems are highly discrete and specialized and thus regulate specific behaviors (Lebestky et al., 2009; Saper et al., 2010; Yokogawa et al., 2012) or are functionally redundant and distributed to protect against localized damage (Pfaff and Banavar, 2007; Pfaff et al., 2008). Finally, our gain-of-function assays will need to be complemented by loss-of-function approaches that reveal the essential roles of these and other neuropeptides.

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