**Drosophila** carboxypeptidase D (SILVER) is a key enzyme in neuropeptide processing required to maintain locomotor activity levels and survival rate

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**Abstract**
Neuropeptides are processed from larger preproproteins by a dedicated set of enzymes. The molecular and biochemical mechanisms underlying preproprotein processing and the functional importance of processing enzymes are well-characterised in mammals, but little studied outside this group. In contrast to mammals, *Drosophila melanogaster* lacks a gene for carboxypeptidase E (CPE), a key enzyme for mammalian peptide processing. By combining peptidomics and neurogenetics, we addressed the role of carboxypeptidase D (dCPD) in global neuropeptide processing and selected peptide-regulated behaviours in *Drosophila*. We found that a deficiency in dCPD results in C-terminally extended peptides across the peptidome, suggesting that dCPD took over CPE function in the fruit fly. dCPD is widely expressed throughout the nervous system, including peptidergic neurons in the mushroom body and neuroendocrine cells expressing adipokinetic hormone. Conditional hypomorphic mutation in the dCPD-encoding gene *silver* in the larva causes lethality, and leads to deficits in starvation-induced hyperactivity and appetitive gustatory preference, as well as to reduced viability and activity levels in adults. A phylogenomic analysis suggests that loss of CPE is not common to insects, but only occurred in Hymenoptera and Diptera. Our results show that dCPD is a key enzyme for neuropeptide processing and peptide-regulated behaviour in *Drosophila*. dCPD thus appears as a suitable target to genetically shut down total neuropeptide production in peptidergic neurons. The persistent occurrence of CPD in insect genomes may point to important further CPD functions beyond neuropeptide processing which cannot be fulfilled by CPE.

**KEYWORDS**
direct mass spectrometric profiling, fruit fly behaviour, M14 carboxypeptidases, peptidomics, proprotein processing
Neuropeptides and peptide hormones are synthesised as parts of larger precursors (preproteins) from which they are sequentially processed into their bioactive form by sequential action of proprotein convertases (PCs), carboxypeptidases (CPs) and amidating enzymes (Fricker, 2005; Zhou, Webb, Zhu, & Steiner, 1999). In mammals, proprotein processing is well-characterised on the molecular and biochemical level, and is implicated in a variety of physiological and pathological processes including obesity and growth defects (Seidah & Prat, 2012; Taylor, Van de Ven, & Creemers, 2003). In contrast, the mechanisms and functions of proprotein processing are little studied in lower vertebrate taxa and invertebrates.

For insects, proprotein processing is best understood for Drosophila melanogaster (reviewed by Pauls et al., 2014). In the fruit fly, the first processing step is catalysed by the proprotein convertase dPC2 encoded by the gene amontilado (amon; Rayburn et al., 2003; Siekhaus & Fuller, 1999; Wegener, Herbert, Kahnt, Bender, & Rhea, 2011). A deficiency in dPC2 results in reduced or absent levels of neuro- and enteroendocrine peptides (Reiter et al., 2011; Rhea, Wegener, & Bender, 2010; Wegener et al., 2011), developmental defects and impaired behaviour including hatching and ecysis (Rayburn, Rhea, Jocoy, & Bender, 2009; Rayburn et al., 2003; Siekhaus & Fuller, 1999) and larval locomotion (Wegener et al., 2011) as well as carbohydrate metabolism (Rhea et al., 2010).

As a typical PC, dPC2 cuts C-terminal of canonical mono- or dibasic amino acid (typically R, KR or RR) cleavage sites, resulting in peptides which are C-terminally extended by basic amino acids. In mammals, these basic C-terminal extensions are pruned by N/E metalloenzyme (EC 3.4.17.10; Che & Fricker, 2002; Che et al., 2001; Fricker & Snyder, 1982), which initially was thought to be the only carboxypeptidase involved in neuropeptide processing (Fricker, 2013a). However, a further member of the M14B subfamily of metalloenzyme (Fricker, 2013a), was later found to be able to partially compensate for a loss of CPE action (Song & Fricker, 1995). CPDs are unusual in that they are large proteins (~180 kDa) that contain three CP domains. Only the first two domains are enzymatically active, while the third domain is inactive (Fricker, 2013a; Garcia-Pardo et al., 2017). In contrast, CPDs and other M14B CPs like the membrane-bound CPM (EC 3.4.17.12) are smaller (52–56 kDa) and contain only one CP domain (Fricker, 2013b; Zhang & Skidelberg, 2013).

Curiously, unlike vertebrates and most other invertebrates, Drosophila lacks a CPE gene and only possesses a CDP-encoding gene named silver (Settle, Green, & Burtis, 1995). The two active domains of dCPD differ in their pH optima and substrate preferences (Sidelyeva, Baker, & Fricker, 2006; Sidelyeva & Fricker, 2002; Sidelyeva et al., 2010). While null mutations in svr are lethal (Bourbon et al., 2002), dCPD transgenes containing either active domain 1 or domain 2 can rescue behavioural and developmental defects of svr mutant flies to varying degrees (Sidelyeva et al., 2010). Hypomorphic mutants have defects in cuticle mela- nisation and tanning, wing morphogenesis, biogenic amine metabolism, light response (Lindsley & Grell, 1968; Wright, 1987) and memory formation (Lu et al., 2016; Sidelyeva et al., 2010), and show increased ethanol and cold sensitivity (Sidelyeva et al., 2010).

The general requirement of CPs in neuropeptide and peptide hormone processing and the lack of a CPE gene in the Drosophila genome imply that dCPD is functionally important for the production of bioactive peptides in the fruit fly. This reasoning is supported by a prominent expression of svr in the central nervous system (CNS) and endocrine tissues such as the midgut (Chintapalli, Wang, & Dow, 2007), as well as by the direct transactivation of svr by the basic helix–loop–helix transcription factor DIMMED which confers a neuroen-docrine peptidergic phenotype (Hadžić et al., 2015). Ectopic expression of svr transgenes in the neurohemal organ of the brain (corpora cardiaca) also affects processing of adipoki- netic hormone (AKH; Sidelyeva et al., 2010). Yet, direct and comprehensive biochemical evidence for a role of dCPD or any other CPD in neuropeptide processing is lacking.

Here, we used a combined neurogenetic–peptidomic approach to test the requirement of dCPD for neuropeptide processing, locomotor behaviour and life span. A phylogenomic search for CPD and CPE genes throughout the insects suggests that CPE has been independently lost in a few holome- tabolous insect taxa, while all analysed insect species likely possess CDP. Taken together, our results suggest that insect CPDs can fully compensate for a loss of CPE.
the 5'-2,174 bp and the 3'-2,351 bp part of the svr (1A-2-3-t2) construct. Then, the pCaSpeR-hs vector (Thummel & Pirrotta, 1991) was digested using EcoRI and XbaI, and the 3'-2,351 bp part was inserted. After ligation, the resulting plasmid was digested with EcoRI, and the 5'-2,174-bp fragment was inserted and ligated. The correct orientation of the fragments and sequence was confirmed by sequencing, and the resulting hs-svr construct (1A-2-3-t2) was introduced into the germline of w1118 flies by BestGene using standard P-element transformation. Several independent transformation lines were obtained which were used to generate w1118; hs-svr1A-2-3-t2 and yw P[w+mWhs=GawB]svrP33/FM7h; hs-svr1A-2-3-t2 lines.

2.3 | Heat-shock rescue

Eggs of yw P[w+mWhs=GawB]svrP33/FM7h; hs-svr1A-2-3-t2 flies were collected every morning, kept at room temperature and heat-shocked daily for 30 min at 37°C in a water bath. Larvae for the behavioural assays were obtained by heat shocking for 4 days, followed by two days at 18°C to minimise background expression of hs-svr. Homozygous mutant larvae were identified by the light mouthparts and unpigmented denticle bands due to the y allele, while larvae carrying FM7h showed stronger pigmentation due to the y allele of FM7h (Brehme, 1941). To obtain adults, heat shocking was continued until eclosion. After eclosion, flies were kept at 18°C for the times indicated. Then, male flies were anaesthetised on ice and nervous tissue was dissected and processed as described below. FM7h controls were distinguished from svrP33 mutants by the presence of bar (B) and orange-coloured eyes.

2.4 | RT-PCR

To test for background expression of hs-svr, total RNA was extracted from heads of five adult males per genotype using the Quick-RNA MicroPrep Kit (Zymo Research) according to manufacturer's instructions. Heads were cut off, collected in a microtube containing 300 μl RNA lysis buffer on ice and homogenised with a plastic pestle. Total RNA was eluted in 8 μl RNase-free water. For cDNA synthesis, the QuantiTect Reverse Transcription Kit from Qiagen was used. All steps were performed following the manufacturer's protocol. Genomic DNA was removed by adding 1 μl of gDNA wipeout buffer to 6 μl of the eluted RNA. Following incubation at 42°C for 5 min, the samples were placed for 2 min at 4°C and 3 μl of a mastermix composed of 8 μl RT Buffer, 2 μl RT Primer Mix and 2 μl reverse transcriptase was added. Reverse transcription was performed for 30 min at 42°C, followed by 3 min at 95°C and 2 min at 4°C. Finally, 40 μl of water was added and cDNA samples were stored at −20°C.

cDNA was PCR-amplified using a JumpStart REDTaq ReadyMix (Sigma-Aldrich) and svr-, GawB- and Gaw-svr-specific primers (see Table S1). α-tubulin was used as internal control. The PCR programme consisted of 5 min at 95°C, followed by 30 cycles of 30 s at 95°C; 30 s at 60°C and 60 s at 72°C, and followed by a final extension for 5 min at 72°C.

2.5 | Direct peptide profiling via MALDI-TOF mass spectrometry

Direct peptide profiling was carried out according to our standard protocol (Wegener, Neupert, & Predel, 2010). In brief, the brain and ventral ganglion (VG) of 14-day-old adult male flies were dissected in HL3.1 saline (Feng, Ueda, & Wu, 2004). Using microscissors, the brain was further divided into optic lobes or central brain. Tissues were then transferred by pulled glass capillaries to a stainless steel MALDI target, remaining saline was removed and the tissues were let to dry. In case of excess salt deposits, a small droplet of ice-cold water was added to the dried tissues and removed after about 1 s to desalt the sample.

Then, 200 nl matrix (saturated solutions of recrystallised α-cyano-4-hydroxycinnamic acid [CHCA]; Sigma) in 30% MeOH: 30% EtOH and 40% water (v:v:v) was added per tissue and let to dry. MALDI-TOF mass spectra were acquired in positive ion mode on an Applied Biosystems 4800 Plus MALDI-TOF/TOF mass spectrometer (Applied Biosystems/MDS Sciex). Samples were analysed in positive reflector mode within a mass window of 850–3,000 Da. Some mass peaks were additionally fragmented in MS/MS mode using PSD. Laser power was adjusted manually to provide optimal signal-to-noise ratio. Raw data were analysed using Data Explorer 4.10 software (Applied Biosystems/MDS Sciex).

2.6 | Sample preparation for NanoLC-ESI-MS/MS

After eclosion, flies were kept for 5 days without heat shock. Then, brains and ventral nerve cords of males were dissected in HL3.1 saline on ice and transferred by a needle to a frozen microtube in a laptop cooler. Per sample, 30 brains plus ventral nerve cords were pooled and stored at −80°C until extraction. For extraction, 50 μl of a methanol, water and trifluoroacetic acid mixture (90/9/1 v/v/v) was added to the frozen tubes, followed by 3 min in an ice-cold ultrasonic bath and 30 min of incubation on ice. Afterwards, the samples were centrifuged at 15,000 g (Hettich Mikro), and the supernatant was transferred to a new microtube and vacuum-dried (Uniequip Univapo 100H).

The extracts were prepurified on self-made StageTips (Rappisilber, Mann, & Ishihama, 2007) using 3M Empore C18 material (ChromTech Inc.) and 200-μl pipet tips. The columns were activated with 50 μl of 100% acetonitrile and
equilibrated with 50 µl of 10 mM HCl. The extracted samples were dissolved in 30 µl of 10 mM HCl, sonicated in an ultrasound bath and applied onto the equilibrated StageTips. After washing with 50 µl 10 mM HCl, peptides were eluted with 30% acetonitrile and dried in a vacuum concentrator (SpeedVac, Eppendorf). Low-binding plastic was used throughout.

### 2.7 NanoLC-MS/MS analysis

Peptides were dissolved in 2% acetonitrile and 0.1% formic acid. NanoLC-MS/MS analyses were performed on an Orbitrap Fusion (Thermo Scientific) equipped with a PicoView Ion Source (New Objective) and coupled to an EASY-nLC 1000 (Thermo Scientific). Peptides were loaded on capillary columns (PicoFrit, 30 cm × 150 µm ID, New Objective) self-packed with ReproSil-Pur 120 C18-AQ, 1.9 µm (Dr. Maisch) and separated with a 30-min linear gradient from 3% to 40% acetonitrile and 0.1% formic acid and a flow rate of 500 nl/min.

Both MS and MS/MS scans were acquired in the Orbitrap analyzer with a resolution of 60,000 for MS scans and 15,000 for MS/MS scans. A mixed ETD/HCD method was used. HCD fragmentation was applied with 35% normalised collision energy. For ETD, calibrated charge-dependent ETD parameter was applied. A Top Speed data-dependent MS/MS method with a fixed cycle time of 3 s was used. Dynamic exclusion was applied with a repeat count of 1 and an exclusion duration of 10 s; singly charged precursors were excluded from selection. Minimum signal threshold for precursor selection was set to 50,000. Predictive AGC was used with AGC, a target value of 2e5 for MS scans and 5e4 for MS/MS scans. EASY-IC was used for internal calibration.

In total, three (controls) and two (mutants) biological samples were measured in technical duplicates. All chemicals used were of HPLC grade; low-binding plastic was used throughout.

### 2.8 Data analysis

Data analysis was performed with PEAKS Studio 8.5 (Bioinformatics Solutions Inc.; Zhang et al., 2012). Parent mass tolerance was set to 8 ppm, and fragment mass tolerance to 0.02 Da. Pyro-Glu (N-term Q), oxidation (Met), carbamidomethylation (Cys) and amidation (C-term) were allowed as variable modification. A maximum number of five modifications per peptide were allowed. Searches were performed against a custom neuropeptide database that contained all known Drosophila and suggested propeptides, processing enzymes and neuropeptide receptors. Results were filtered to 1% PSM-FDR. To calculate the processing index (PI), we first calculated the mean ratio R of the peak area (PA) between the fully processed and unprocessed form \( R = \frac{PA_{\text{fully processed}}}{PA_{\text{C-term extended}}} \) for the mutant and control samples, respectively. These ratios were then used to calculate the PI for each peptide \( \text{PI} = \frac{R_{\text{mutant}}}{R_{\text{control}}} \) which corrects for possible differences in the ionisation probabilities between processed and unprocessed peptides. In case a peptide was only detectable in its unprocessed form in mutants leading to a division by 0, the PI was set to \( 10^{-10} \). Likewise, if a peptide was only detected in its processed form in the mutant but not the control, the PI was set to 2.

### 2.9 Immunostainings

\( svf^{PG33} / FM7h \) flies were crossed with \( w^* \), \( 10 \times UAS-IVS-myrGFP \) or \( w^* ; UAS-Stinger \). L3 larval CNS of the F1 progeny 2 days after last heat shock was dissected in HL3.1 saline (Feng et al., 2004) and fixed for 2 hr in 4% paraformaldehyde in 0.1 M PBS at room temperature. Then, the CNS was washed in 0.1 M PBS with 0.3% Triton X (PBT) and incubated in PBT + 5% normal goat serum for 1 hr at room temperature. Afterward, primary antibodies (anti-GFP rabbit polyclonal [1:1,000; Invitrogen] and anti-brp [nc82 mouse monoclonal [mAb; 1:100]) in PBT + 5% NGS, were added and preparations were incubated overnight at 4°C on a shaker. Preparations were then washed 5× for at least 1 hr in 1× PBS at room temperature. Fluorophore-coupled secondary antisera (goat anti-rabbit Alexa 488 and goat anti-mouse DyLight 649; Dianova GmbH), diluted 1:1,000 in PBT + 5% NGS, were added and preparations were incubated overnight at 4°C on a shaker. Next, preparations were washed as above, followed by a final wash in 0.1 PBS and mounting in 80% glycerol in 0.1 M PBS. The preparations were analysed with a Leica TCS SPE confocal microscope (Leica SPE, Leica Microsystems), using ACS APO 20×/0.60 and ACS APO 40×/1.15 objectives. Images were processed with Fiji (Schindelin et al., 2012) and Adobe Photoshop CS6 (Adobe Systems; V.6.0.1).

### 2.10 Locomotor activity recording

Four- to seven-day-old flies were recorded individually with the Drosophila Activity Monitoring (DAM; TriKinetics) system. To record normal rhythmic behaviour, flies were kept in glass tubes with 2% agarose and 4% sugar on one side under standard 12:12 light:dark conditions with light intensities around 100 lux at 20°C and 60% humidity. Fly activity was defined by the number of infrared light beam crosses per minute or per day. Under starved conditions, flies were kept on 2% agarose to avoid dehydration.

### 2.11 Survival

To test flies for changes in life span, groups of ten flies were kept under normal ad libitum standard food conditions at
25°C and 60% humidity and dead flies were counted at the end of each day. Flies were transferred onto fresh food every 3–4 days to circumvent influences due to changes in food quality.

2.12 | Larval preference tests

To test larvae for innate odour and taste preference, the FIM (ETIR-based Imaging Method) tracking system (Risse et al., 2013) was used to monitor individual larvae over time. Recordings were made by a monochrome industrial camera (DMK27BUP031) with a Pentax C2514-M objective in combination with a Schneider infrared pass filter, and the IC capture software (www.imagingsource.com). Larval position in relation to the odour or taste stimulus was determined every second. To test larvae for innate odour responses, a thin layer of 1.5% agarose was placed on an acryl plate which was illuminated with infrared light. An odour container (10 μl amyl-acetate) was placed on one side of the agarase layer. A group of five larvae was placed in the neutral midzone of the agarose layer, and larvae were recorded for 5 min. To monitor larval responses to fructose or high salt, larvae were placed on a 1.5% agarose layer which contained either 2 M fructose or 1.5% sodium chloride on one side.

Preference indices (PIs) were calculated by subtracting the number of larvae on the stimulus side (ST) from the number of larvae on the no-stimulus side (NO), divided by the total number of larvae: PI = (#ST – #NO)/#TOTAL. Negative PI values indicate avoidance behaviour, while positive PI values indicate approach behaviour.

2.13 | Phylogenomic analysis

A genome-based tblastn search on the NCBI and i5k workspace@NAL website was performed, using the protein sequences for Drosophila CPD and Caenorhabditis elegans CPE as a query. The search was then repeated with identified putative insect CPE sequences. Incomplete hits were removed, resulting in a list of 313 CPE/D/M-like sequences that were analysed using MEGA X (Kumar, Stecher, Li, Knyaz, & Tamura, 2018). First, sequences were aligned using the Muscle algorithm, and then a maximum-likelihood tree employing the JTT matrix-based amino acid substitution model (Jones, Taylor, & Thornton, 1992) with all sites was calculated with a bootstrap value of 500.

2.14 | Statistical analysis

Data were tested for normal distribution using a Shapiro–Wilk test. For the comparison of genotypes, an unpaired two-sided t test (pairwise.t.test) was used for normally distributed data and an unpaired Wilcoxon rank-sum test for nonparametrically distributed data. Statistical analyses were done with RStudio, Version 1.0.136 (www.r-project.org). Significance levels between genotypes shown in the figure refer to raw p-values obtained in the statistical tests.

3 | RESULTS

3.1 | Characterisation of the transgenic heat-shock rescue lines

The svrPG33 allele was generated via a genomic P-element mediated PGawB insertion into the second exon of svr on the first (X) chromosome (Bourbon et al., 2002). The insertion inhibits expression of svr mRNA (Sidelyeva et al., 2006) leading to embryonal and early larval lethality in hemizygous males and homozygous females. To rescue lethality, we generated transgenic flies carrying a hs-svr1A-2-3-t2 genomic insert, which under the control of a heat-shock (hs) promoter expressed a transgenic and endogenously expressed CPD form consisting of an inactive first and third CP domain, and an active second CP domain, a transmembrane domain and the tail-2 region (Sidelyeva et al., 2010). To test the functionality of the newly generated hs-svr rescue lines, we crossed w1118/svrPG33/FM7h virgins with males of two different FM7/Y; hs-svr lines and kept the flies at constant 18°C or under a heat-shock regime. We then scored the F1 adult males for the dominant FM7 marker Bar (B). At constant 18°C, only B males (FM7/Y; hs-svr+) were present in both crosses (59/40 males, respectively), confirming the early lethality of the homozygous svrPG33 genotype. Under heat-shock regime, both B and normal-eyed males were found (36:33/37:13, respectively), indicating a ≥35% heat-shock rescue efficiency for svrPG33/Y males. Thus, the newly generated hs-svr lines are functional and can be used to rescue lethal svr mutations.

While a hs-promoter is ideal to drive rescue gene expression in nonfeeding developmental stages (egg, pupa), it is prone to low background expression at normal temperatures (Steller & Pirrotta, 1985). Using RT-PCR with svr-specific primers (Figure S1a), we found svr expression in w1118 controls containing a functional svr gene, in heat-shock-rescued svrPG33, hs-svr mutant flies, and in svrPG33, hs-svr flies maintained at 18°C (Figure S1b). To distinguish whether the svr expression in svrPG33, hs-svr flies at low temperature is due to background expression from the hs transgene or expression from the endogenous svrPG33 allele, we performed RT-PCR with a GawB/svrPG33 insert-specific primer set (Figure S1c). GawB expression was absent from w1118 controls, but detectable in heat-shock-rescued svrPG33 mutant flies. Again, a weak band was found for svrPG33, hs-svr flies maintained at 18°C. A primer set spanning the region from the 3’ end of GawB to svr exon 5 did not result in a detectable product in all flies and conditions tested. This confirms previous findings that the PG33 insertion disrupts expression

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FIGURE 1 MALDI-TOF spectra from direct peptide profiling. (a) Example for central brain tissue of a FM7h; hs-svr control fly in the mass range of 900–2,000 Da. Note that all detected neuropeptides are fully processed, indicated by black letters. (b) Example for central brain tissue of a svrPG33; hs-svr mutant fly. Note that the profile for fully processed neuropeptides (black letters) is very similar to that for controls in (a). Yet, in addition, peaks indicating C-terminally extended forms (red letters) are present. (c) Similar C-terminally extended forms are also visible in other tissues, here a ventral nerve cord of a svrPG33; hs-svr mutant fly although a partly different neuropeptide complement is present.
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<th>Peptide</th>
<th>sequence fully processed</th>
<th>sequence C-terminally extended</th>
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(Continues)
of native svr mRNA (Sidelyeva et al., 2006), and indicates that svr<sup>PG33</sup> mutant flies do not express a functional native dCPD. However, the hs-svr rescue construct obviously has a low background expression even at 18°C. Without heat shock, svr<sup>PG33</sup>; hs-svr flies thus likely do not represent full dCPD knockouts.

### 3.2 Deficiency of dCPD SILVER results in incompletely processed neuropeptides and peptide hormones

To qualitatively test whether a defective dCPD results in changes in peptide processing, we raised svr<sup>PG33</sup>/FM7h; hs-svr flies under heat-shock conditions until eclosion, followed by fourteen days at constant 18°C. Then, the nervous system of individual males was dissected and either optic lobes (<i>n</i> = 15 controls/17 mutants), central brain without optic lobes (<i>n</i> = 12/16) or the ventral ganglion (<i>n</i> = 14/9) were separated and directly profiled by MALDI-TOF MS (Figure 1). This method comprises on-plate extraction, which specifically extracts peptides (Schachtner, Wegener, Neupert, & Predel, 2010; Wegener et al., 2010) and allows to reliably identify peptides by mass match in <i>Drosophila</i> (Predel et al., 2004; Wegener, Reinl, Jänsch, & Predel, 2006) and other insects (e.g. Audsley, Matthews, Down, & Weaver, 2011; Diesner et al., 2018).

In total, direct peptide profiling by mass match (±0.5 Da) identified bioactive and C-terminally extended forms of 43 different neuropeptides (Table 1). These peptides represent about 80% of the <i>Drosophila</i> neuropeptides in the CNS confirmed by combined mass spectrometry in earlier studies (direct peptide profiling; Predel et al., 2004; Wegener et al., 2006) or LC-MS (Baggerman, Boonen, Verleyen, De Loof, & Schoofs, 2005; Baggerman, Cerstiaens, De Loof, & Schoofs, 2002; Yew et al., 2009). The obtained direct peptide profiles in controls matched nicely with that of previous reports (Predel et al., 2004; Wegener et al., 2006) which did not find C-terminally extended neuropeptides in the CNS of wild-type flies.

In <i>FM7h</i>; hs-svr control flies, 35 of the detected peptides were only found in their processed bioactive form (Table 1, Figure S2). Five peptides (CAPA-PVK-2, CCHA-2, FMRFa-3, FMRFa-4 and kinin) occurred in both processed bioactive and C-terminal extended forms. For AstA-2 and CCAP, only mass peaks matching the unprocessed peptide carrying a C-terminal GKR and GRKR extension were identified. Due to the prevalence of nonpolar amino acids in AstA-2 and CCAP (carrying an internal cysteine bridge), both peptides are usually difficult to detect by direct MALDI-TOF peptide profiling without prior chemical modification. We assume that the C-terminal Arg of the unprocessed peptide likely increased ionisation efficiency during the MALDI process (Krause, 1999). In 99.0% of the detected peptide
peaks over all preparations, fully processed forms were found, while peptides carrying a C-terminal basic amino acid extension occurred in only 5% of the peptide peaks over all preparations (Figures 2 and S2).

In svrPG33, hs-svr experimental flies, 37 neuropeptides were detectable by mass match in both processed bioactive and C-terminal extended form. To test the validity of the mass matching, we sequenced masses matching APK-R, AstA-4, CAPA-PK(2-15)-GKR, CAPA-PVK-1, corazonin-GKR, FMRFa-2-GR, FMRFa-3-GR, FMRFa-4-GR, FMRFa-6, FMRFa-6-GR, HUG-PK-GKR, IPNa-GKR, myosuppressin-GRR, SIFa and sNPF(4-11)-GR by MS/MS fragmentation and consistently confirmed their supposed identity (Table 1). Five neuropeptides (Ast-C, CCHa-2, FMRFa-8, MIP-3 and MIP-5) were exclusively found in their processed bioactive form. Four neuropeptides (CCAP, kinin, MIP-2 and PDF) were exclusively found in their unprocessed C-terminally extended form (Figure S2). 76.8% of the mass peaks corresponded to fully processed forms, and 43.2% matched peptides with C-terminal basic amino acid extension (Figures 2 and S2). The qualitative results obtained by direct peptide profiling indicate that the occurrence of unprocessed C-terminally extended neuropeptides is highly increased across the peptidome in svrPG33, hs-svr flies, suggesting that dCPD is involved in the processing of most if not all neuropeptides.

To obtain a more quantitative measure, we next used nanoLC-ESI-MS/MS and analysed relative differences in the detection levels of processed and unprocessed peptides in brain extracts of svrPG33, hs-svr mutant flies and FM7h; hs-svr controls. Representative data are shown for allatostatin A and FMRF-like peptides in Figure 3. For each neuropeptide, we calculated a processing index (PI, see material and methods) as a measure of the relative differences in processed vs. unprocessed peptides between svrPG33, hs-svr mutant flies and FM7h; hs-svr controls (Table 1, underlying ratios are shown in Figure S3). A PI = 1 means that the ratio of processed to unprocessed forms of a peptide is unchanged between control and mutant flies, a PI > 1 means that there is more processed peptide in the mutants, while a PI < 1 means there is more unprocessed peptide in the mutant compared with control flies. With exception of MIP-3 (PI = 100), the long forms of sNPF-1 (PI = 1.7) and sNPF-2 (PI = 50.6), DTK-3 (PI = 1.0) and DTK-5 (PI = 1.4), all peptides had a PI < 0.1, confirming that dCPD is required for C-terminal trimming and peptide processing. In FM7h; hs-svr controls, all peptides were exclusively detectable in their fully processed form (R = 100), with exception for AstA-1 and NPLP1-IPNa. Unprocessed AstA-1 was found in one out of three samples (R = 12.5), and unprocessed NPLP-1-IPNa occurred in two out of three samples (R = 12.4 and 1.9). In svrPG33, hs-svr mutant flies, most peptides were found in both processed and unprocessed forms, confirming the results from MALDI-TOF peptide profiling. LC-MS also confirmed the MALDI-TOF detection of exclusively unprocessed kinin, MIP-2 and PDF in the mutant (PI = 10^{-10}). Also corazonin, MIP-1 and MIP-4 were exclusively detectable in their unprocessed form. Further, MIP-3 but not MIP-5 and FMRFa-8 was found in exclusively processed form in the mutant. Like MIP-3, also the long forms of sNPF-1 (sNPF-1(1-11)) and sNPF-2 (sNPF-2(1-19)) were only detectable in the mutant, but occurred with a smaller peak area also in the unprocessed forms. This explains the positive PI s for these peptides (Table 1). The presence of only processed MIP-3 in mutant flies is difficult to account for. sNPF-1(1-11) and sNPF-2(1-19) have been found with lower signal intensity in previous neuroepidemiologic studies (Baggerman et al., 2002, 2005; Nässel, Enell, Santos, Wegener, & Johard, 2008; Predel et al., 2004; Wegener et al., 2006; Yew et al., 2009). The existence of the long form sNPF-2(1-19) is shown here for the first time, although this peptide was predicted from the Drosophila genome (Vanden Broeck, 2001). Possibly, the proper intrapeptide processing at monobasic arginine sites to yield the sequence-identical short sNPF forms (sNPF-4-11, sNPF-2(12-19)) requires dCPD trimming. Taken together, the results of direct mass spectrometric MALDI-TOF profiling...
Allatostatin A

1  MNSLNAHLLLLAVCCCGYIAASSPVIGQDQRGSDASDVLAADEADNGDGONIDPAVRYAPGGLSRRAYMYTNGFQMDRFPLFVYNFGLAERKRFSRFYSFGG

**FM7h;hs-svr run 1**

1  KRSDYDYDQDEIDYRVSPPANLVAERRVARVPGQNGRQTRFQPFFNFLGRR

**svr<sup>P33</sup>;hs-svr run 2**

1  KRSDYDYDQDEIDYRVSPPANLVAERRVARVPGQNGRQTRFQPFFNFLGRR

FMRFa-like peptides

1  MGIAYYFLLALYQMQASAIEIHTFHYAHSNLQRDAEDSEVSFGQQNLVDALOLODQTREAELEFRHPISVIGIDYKSKNMLFMKQGRKPRYKDPHEL

**FM7h;hs-svr run 1**

1  EVQONFMRKQGKEDUAEQVLSDEPLEGMAABACDRYGRDFKQDFRFGDFRAEDFMFRGDSNFQVRSNPQFDNFRMPGSAFQOVFGSNSDFHFRMRKLKPAAPFESKFVSMQGQ

**svr<sup>P33</sup>;hs-svr run 3**

1  EAFRRSVQDNFMFGRKQGKEDUAEQVLPSDEPLEGMAABACDRYGRDFKQDFRFGDFRAEDFMFRGDSNFQVRSNPQFDNFRMPGSAFQOVFGSNSDFHFRMRKLKPAAPFESKFVSMQGQ

- amidation (-0.98)
- oxidation (M) (+15.99)
- pyro-Glu from Q (17.03)
and LC-ESI-MS indicate that dCPD is a key enzyme in neuropeptide processing.

3.3 | svr is broadly expressed throughout the larval and adult CNS

The peptidome-wide impairment of neuropeptide processing in svr<sup>PG33</sup>, hs-svr mutants suggested that dCPD is broadly expressed in peptidergic neurons throughout the CNS. To test this, we made use of the intragenic Gal4 sequence contained within the inserted enhancer-trap element PGawB of svr<sup>PG33</sup> (Bourbon et al., 2002) and drove the expression of either a membrane-bound or nuclear form of UAS-GFP. In both the larval and adult CNS, a large number of neurons innervating basically all neuropiles expressed svr<sup>PG33</sup>-Gal4-driven GFP (Figure 4a). This is in line with the idea that many Drosophila neurons express peptide co-transmitters (Nässel, 2018).

**FIGURE 3** Examples of the LC-MS peptide analysis for two peptide families, allatostatin A and FMRFa-like peptides. Identified peptides were aligned to the prepropeptide sequence. For both peptide families, fully processed and bioactive peptides are C-terminally amidated (red box); both spacer peptides and bioactive peptides were found. In FM7h; hs-svr control flies, only fully processed amidated bioactive peptides or mostly processed but not yet amidated (still carrying a C-terminal glycine amidation signal) are visible, with exception for AstA-1 (VERYAFGLa) which seems to have a weak C-terminal PC cleavage site. In svr<sup>PG33</sup>, hs-svr mutant flies, additional C-terminally extended peptides become detectable which still comprise the C-terminal PC cleavage sequence (KR, RR, R). Interestingly, a mutated carboxypeptidase D (dCPD) leads sometimes to a skip of PC cleavage between some directly neighboured bioactive peptide sequences in both peptide families. Perhaps, intermediate dCPD action is required between two consecutive PC cleavage events in these cases.

**FIGURE 4** svr expression pattern based on the intragenic PG33 insert. The Gal4 sequence contained within the PG33 GawB insert was used to drive either a membrane-bound (myr::GFP, a) or nuclear (Stinger) form of GFP (b–d) in the larval central nervous system. Staining with mAb nc82 against the synaptic protein bruchpilot was used to mark neuropil areas (a”–d”). Maximum projections of confocal stacks. (a) The whole neuropil is uniformly innervated by svr<sup>PG33</sup>-expressing neurons, only the densely packed mushroom bodies (MB) stick out. In the attached ring gland, the endocrine cells producing adipokinetic hormone are also strongly labelled (asterisk). (b) The nuclear staining shows that many but by far not all neurons express svr<sup>PG33</sup>. Again, the Kenyon cells forming the mushroom bodies (MB) as well as the adipokinetic hormone (AKH) cells (asterisks) are conspicuous. (c) Close-up of the MB shown in (b) shows the densely packed Kenyon cell somata (asterisk) in the dorsal protocerebrum. (d) Close-up of the AKH cells in (b), located in the corpora cardiaca portion of the ring gland.
Due to the broad expression, it was impossible to identify specific arborisation patterns except for the tightly packed Kenyon cells with their parallel projections in the mushroom bodies (Figure 4a–c). These neurons express sNPF (Johard et al., 2008; Nässel et al., 2008). As sNPF processing was impaired in svrPG33, hs‐svr flies (Table 1, Figure S2), dCPD likely plays a role in peptide processing in these higher order integration centres. Outside of the CNS, the conspicuous endocrine AKH cells in the glandular part of the corpora cardiaca and the proximal part of the larval ring gland were strongly labelled (Figure 4a,d). This is compatible with the finding that genetic manipulations of dCPD levels in these cells significantly affect AKH processing (Sidyelyeva et al., 2010).

FIGURE 5 Carboxypeptidase D is required for starvation‐induced hyperactivity and affects survival rate and locomotor activity. (a) svrPG33, hs‐svr mutant flies showed an early onset of starvation‐induced hyperactivity (~20 hr) during the first day without food. In contrast, control flies started starvation‐induced hyperactivity later, during the second night after ~30 hr of starvation. (b) Statistic comparison of averaged locomotor activity for the first two nights and days after starvation onset. n = 23–29. (c) Survival is significantly lower between day 18 and day 45 in svrPG33, hs‐svr mutant flies compared with control flies. (d) Total locomotor activity in svrPG33, hs‐svr mutant flies is significantly reduced (p = 5 × 10−14) compared with control flies, especially during morning and evening activity bouts. Yet, daily rhythmicity remains unaffected. n = 53–61.

Significance level *p < .05, **p < .01, ***p < .001

3.4 | dCPD is required for starvation‐induced hyperactivity

The expression pattern of svrPG33 suggested that dCPD is expressed in AKH‐producing endocrine cells of the corpora cardiaca. AKH has metabolic functions and is required for starvation‐induced hyperactivity (Lee & Park, 2004; Yu et al., 2016). To address the functional requirement of dCPD in AKH processing, we tested whether starvation‐induced hyperactivity is affected in svrPG33, hs‐svr mutant flies (Figure 5a,b). At ZT 12 (lights off), flies were placed into monitor tubes containing either only agarose (starvation condition) or agarose and sucrose (control condition). All genotypes showed significant starvation‐induced hyperactivity during the experiment on agarose (Figure 5a). svrPG33, hs‐svr mutant flies
increased their locomotor activity already during the first day (12–24 hr of starvation), while controls still showed normal day activity. During the following night (24–36 hr of starvation), also control larvae increased their locomotor activity to a significant level (Figure 5b). The maximal levels of activity of svrPG33, hs-svr mutant flies were quite stable throughout the 72 hr under starvation, and locomotor activity still showed daily rhythmicity with distinguishable peaks during morning and evening and intermediate siesta phase from 24 to 60 hr. In contrast, locomotor activity of control flies peaked during the second night after 24–36 hr of starvation, and then steadily declined until all flies had died at 72 hr without any sign of a siesta phase. Thus, svrPG33, hs-svr mutant flies showed reduced hyperactivity compared with controls, in which starvation-induced hyperactivity completely overruled the daily activity pattern. This phenotype is compatible with the assumption of a hypomorphic alteration of AKH processing.

3.5 | Loss of dCPD affects life span and general activity levels

We next assessed the general health state of svrPG33, hs-svr mutant flies by analysing their locomotor activity level and life span. Both svrPG33, hs-svr mutant and control flies showed a maximum life span of around 50 days under ad libitum feeding conditions at 25°C. Yet, svrPG33, hs-svr mutant flies showed a significantly higher mortality rate between day 18 and day 45 compared with control flies (Figure 5c). Next, we analysed locomotor activity and rhythmicity. Control flies displayed the characteristic bimodal activity pattern with increased morning and evening activity and a siesta phase during mid-day. svrPG33, hs-svr mutant flies showed the same rhythmicity, yet an overall reduced activity pattern, with dampened morning and evening activity bouts (Figure 5d).

3.6 | dCPD is required for appetitive but not aversive gustatory preference

Neuropeptides act both as signalling and as modulatory substances within chemosensory input pathways in Drosophila (Hückesfeld, Peters, & Pankratz, 2016; Root, Ko, Jafari, & Wang, 2011; Shankar et al., 2015; Winther, Acebes, & Ferrús, 2006). We therefore used larval preference assays (Selcho, Pauls, Huser, Stocker, & Thum, 2014) to test whether dCPD-mediated peptide processing plays a role in gustatory or olfactory signalling. First, we tested the preference for fructose, a sugar that provides nutritional value and sweetness (Rohwedder et al., 2012). svrPG33, hs-svr mutant flies showed a reduced preference behaviour for fructose compared with control larvae. (b,e) svrPG33, hs-svr mutant larvae avoided high salt concentration similar to control larvae over the course of time. (c,e) Odour response to amylacetate was indistinguishable between svrPG33, hs-svr mutant larvae and control larvae. n = 30–42. Significance level *p < .05. n.s., not significant.
larvae showed approach behaviour for fructose; however, the performance was reduced compared with control larvae in the course of time (Figure 6a) and significantly reduced after 5 min (Figure 6d). To test whether a lack of dCPD generally affects gustatory responses, we challenged larvae with aversive high salt concentrations. As expected, control larvae significantly avoided high salt concentrations. Similarly, svrPG33, hs‐svr mutant larvae showed avoidance behaviour, suggesting that the lack of dCPD affected the larval response specifically to sugar or appetitive substances and not taste responses in general (Figure 6b,e). Next, we tested larvae for their innate olfaction. svrPG33, hs‐svr mutant larvae and control larvae performed indistinguishable over the course of time and approached amylacetate (Figure 6c,f). Our results suggest that hypomorphic dCPD levels do not affect chemosensory sensory processing in general, but rather gustatory responses to sugar.

### 3.7 Evidence for an independent loss of CPE but not CPD in holometabolous insects

Carboxypeptidase E, the major neuropeptide processing CP in mammals, has also been found in molluscs (Juvvadi, Fan, Nagle, & Fricker, 1997) and nematodes (Husson et al., 2007; Jacob & Kaplan, 2003), but is absent in Drosophila (Sidyelyeva & Fricker, 2002). To test whether this lack of CPE applies to insects in general, we performed a nonexhaustive insect genome BLAST search based on CPE sequences from mouse and C. elegans (EGL-21), and CPD sequences from Drosophila. Obtained insect CPE sequences were then used to refine the search. In total, we obtained 310 predicted full CP sequences from insects covering the major insect orders. This analysis certainly does not include all available sequence information for insects, and we did not specifically search for CP sequences within individual genomes. A maximum-likelihood tree clustered CPE and CPD/CPM separately of each other (Figure S4). CPDs are significantly larger than CPEs and CPMs due to their three CP domains. This criterion was used to separate CPDs from CPMs which otherwise show high sequence similarity between their active CP domains. Our BLAST search yields CPD sequences for most insect orders (Table 2), except for diplurans, mayflies (Ephemeroptera), dragonflies (Odonata) and stick insects (Phasmatodea) for which little genomic data are available and CPD sequences are likely to be found in the future. A conservative interpretation of the data is that all insect orders for which abundant genomic data are available possess CPD (Table 2). CPE was found in the basal Entognatha and in the majority of insect orders including most hemimetabolous orders and the holometabolous beetles and derived Lepidoptera (butterflies, moths and allies) (Table 2). Interestingly, putative CPE sequences could not be identified from those large holometabolous orders which contain the majority of insects with sequenced genomes: the Diptera (mosquitoes, flies and allies) and the Hymenoptera (bees, ants, wasps and allies) (Table 2). Thus, CPE seems to be common throughout the insects, but apparently has been independently lost at least two times: in the basal holometabolous Hymenoptera, as well as the derived holometabolous Diptera. A puzzling finding not concurrent with this notion is the presence of a putative CPE sequence in the snowberry fruit fly Rhagoletis zephyria but not other species belonging to the Tephritidae (true fruit flies, not directly related to the Drosophilidae (vinegar flies)). The size and sequence including the first and second block of Zn2+ binding residues classifies this predicted CP as CPE. We also found a true CPD gene for R. zephyria as well as other tephritid species, speaking against a gene prediction or sequencing error. Further studies are required to confirm and explain the surprising putative presence of CPE in a tephritid.

### 4 Discussion

N/E metallocarboxypeptidases of the M14 family catalyse the second step in prepropeptide processing by removing the C-terminal mono- or dibasic cleavage signal that remains after proprotein convertase cleavage of the propeptide (Fricker, 2013a). Peptidomic studies detected a large and
broad accumulation of C-terminally extended neuropeptides in Cpe\textsuperscript{kr}/ICpe\textsuperscript{kr} mice (Che, Biswas, et al., 2005; Che, Yuan, et al., 2005; Che et al., 2001) and egl-21 mutant C. elegans (Husson et al., 2007). This indicated that CPE is the key carboxypeptidase for neuropeptide processing in these phylogenetically very distant animals. It was thus surprising that the genome of the fruit fly Drosophila lacks a CPE-encoding gene (Sidelyeva & Fricker, 2002; Taghert & Veenstra, 2003), the only insect in which neuropeptide processing is studied in more detail (Pauls et al., 2014). Several lines of evidence indicated that dCPD encoded by svr has taken over the key function of neuropeptide processing in Drosophila. First, the second domain of dCPD has enzymatic properties similar to those of mammalian CPE (Sidelyeva & Fricker, 2002). While mammals have only one main splice form of CPD mRNA and CPD is absent from mature secretory vesicles that contain CPE (Varlamov, Eng, Novikova, & Fricker, 1999; Varlamov & Fricker, 1998), dCPD mRNA has various splice forms. Of these, the long forms carrying tail-1 likely are located mostly to vesicles outside the perinuclear area when expressed in Drosophila and mammalian cell lines (Kalinina, Fontenele-Neto, & Fricker, 2006), mimicking the localisation pattern of mammalian CPE. Next, ectopic expression of svr in the endocrine AKH-producing cells reduced the levels of naturally occurring C-terminally extended AKH (Sidelyeva et al., 2010). Lastly, flies with disrupted svr gene are embryonically lethal (Settle et al., 1995; Sidelyeva et al., 2006) and show deficits in various neuropeptide-regulated behaviours (Sidelyeva et al., 2006). Direct evidence for an involvement of CPD in neuropeptide processing was, however, lacking so far for any species, including the fruit fly. Hence, a main result of our peptidomic study is the demonstration of the ability of dCPD to efficiently process a broad variety of neuropeptides. This raises the possibility that the failure of mouse CPD to fully rescue a deficiency of CPE (Song & Fricker, 1995) is caused by an incomplete overlap of the CPD and CPE expression patterns in peptidergic neurons rather than by a reduced efficiency of mouse CPD to process neuropeptides.

The early lethality of svr mutants (Settle et al., 1995; Sidelyeva et al., 2006) suggests that dCPD function during development cannot be substituted by dCPM. Our finding that svr\textsuperscript{PG33} mutant larvae and pupae required daily heat-shock rescue to develop into adults is in line with the importance of svr during development. Yet, once adult, svr\textsuperscript{PG33} mutants survived without heat shock even though the mean life span was significantly reduced. Our RT-PCR results suggest that this reduced viability is due to a background expression of the hs-svr rescue construct even at lower temperature and not due to a loss of dCPD requirement in adult flies. We had previously observed a similar phenomenon for amon (dPC2)-deficient flies rescued by a hs-amon construct (Wegener et al., 2011). We hypothesise that the production rate of neuropeptides is significantly lower in adult flies than in the very fast growing larva or developing pupa, in which the background expression of either hs-amon or hs-svr may not be sufficient to provide the required increasing supply in neuropeptides during development. Alternatively, adults may better cope with reduced levels of neuropeptides and possible other products of CPD action which presumably are less critical for post-developmental viability, at least under laboratory conditions.

Taken together, our results suggest that dCPD is a key if not the sole CP involved in neuropeptide processing in Drosophila.

As a mutation in either amon or svr is lethal, it is surprising why during evolution Drosophila has kept only one gene for a proprotein convertase (dPC2, amon; Wegener et al., 2011) and one peptide processing CP (CPD, svr, this study), given that most animals have additional genes (PC1/3 and CPE). The fact that CPD and not CPE has been kept could be explained by the potential higher versatility of CPD with its two bioactive domains and membrane-anchored and free isoforms which have unique functions, substrate specificities and specific pH optima (Garcia-Pardo et al., 2017; Sidelyeva et al., 2010). It is tempting to speculate that dCPD has further functions additional to neuropeptide processing, which cannot be executed by CPE with its single bioactive domain. This is in line with the finding that hypomorphic svr mutants have phenotypes in wing morphology (Sidelyeva et al., 2006) and biogenic amine pools (Wright, 1987). This hypothesis is also supported by our finding that obviously CPE has been independently lost at least twice during insect evolution in Hymenoptera and Diptera, while CPD seems to be present in all insect taxa. Functionally, it will be interesting to see whether other insects have various CPD mRNA splice variants and whether the number of variants correlates with the presence of CPE.

Based on the expression pattern of the svr\textsuperscript{PG33} transgene, dCPD is broadly expressed in the CNS and neuroendocrine organs. The expression in the Kenyon cells of the mushroom body, an important brain centre for learning and memory, is in line with impaired long-term memory in a courtship assay (Sidelyeva et al., 2010) and olfactory memory formation (Lu et al., 2016) found in svr hypomorphs. Similarly, knockout of CPE in mice impairs olfactory social learning, object recognition memory and performance in the Morris water maze (Woronowicz et al., 2008). The expression of svr\textsuperscript{PG33} in the AKH cells of the corpora cardiaca, as well as its effect on AKH-mediated starvation-induced hyperactivity, indicates a role of dCPD in AKH processing after dPC2 cleavage (Rhea et al., 2010). This is supported by the effect of ectopically expressed dCPD on AKH processing as shown by direct peptide profiling (Sidelyeva et al., 2010). Impairment in insulin signalling increases life span (Brogiole et al., 2001; Tatar et al., 2001), and dCPD has been implicated in insulin processing (Lu et al., 2016). Yet,
we found the mean life span of the svrPG33 hypomorphs is decreased which suggests a further, unidentified role of dCPD outside of peptide processing or an involvement of other yet unknown neuropeptides in the regulation of life span. These neuropeptides could be identified by cell-specific svr knock-out via CRISPR-Cas9 (Xue et al., 2014) or knockdown via RNAi, as the svrPG33 driver line lends itself as a tool to generally impair peptide signalling in neurons even if these express multiple co-expressed or unknown peptides.

Taken together, our results show that dCPD is a key enzyme for neuropeptide processing in Drosophila, and is required for proper peptide-regulated behaviour. The finding that CPE but not CPD has been independently lost in two major insect taxa may point to important further CPD functions beyond neuropeptide processing which cannot be fulfilled by CPE.

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CONFLICT OF INTEREST

We declare no conflict of interests.

DATA AVAILABILITY STATEMENT

Original data will be archived at Dryad upon publication.

AUTHOR CONTRIBUTIONS

DP and CW designed the study; YH, JTV, AS, JK and CW performed and analysed the mass spectrometric measurements; YH and CW performed immunostainings; DP and LT performed and analysed the behavioural assays; TS and GG created the svr-hs construct; LT and GG performed RT-PCR; CW performed the phylogenomic analysis; and CW and DP wrote the manuscript draft. All authors read and commented on the final manuscript version.

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