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Sequence and expression of the *CAPA*/*CAP2b* gene in the tobacco hawkmoth, *Manduca sexta*

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Summary

The gene coding for cardioacceleratory peptide 2b (CAP2b; pELYAFPRV) has been isolated and sequenced from the moth *Manduca sexta* (GenBank accession #AY649544). Because of its significant homology to the *CAPA* gene in *Drosophila melanogaster*, this gene is called the *Manduca CAPA* gene. The *Manduca CAPA* gene is 958 nucleotides long with 29 untranslated nucleotides from the beginning of the sequence to the putative start initiation site. The *CAPA* gene has a single open reading frame, 441 nucleotides long, that codes for a predicted precursor protein of 147 amino acids. The predicted prepropeptide encodes a single copy of each of three deduced propeptides, a CAP2b propeptide, with a Q substituted for an E at the N-terminus (QLYAFPRVa), and two novel CAP2b-related propeptides (DGVLNLYPFPRVa and TEGPGMWFGPRLa). To reduce confusion and to adopt a more standardized nomenclature, we rename pELYAFPRVa as Mas-CAPA-1 and assign the names of Mas-CAPA-2 to DGVLNLYPFPRVa and Mas-PK-1 (Pyrokinin-1) to TEGPGMWFGPRLa.

The spatial and temporal expression pattern of the *CAPA* gene in the *Manduca* central nervous system (CNS) was determined in all major post-embryonic stages using *in situ* hybridization techniques. The *CAPA* gene is expressed in a total of 27 pairs of neurons in the post-embryonic *Manduca* CNS. A total of 16 pairs of cells is observed in the brain, two pairs in the sub-esophageal ganglion (SEG), one pair in the third thoracic ganglion (T3), one pair in each unfused abdominal ganglion (A1–A6) and two pairs in the fused terminal ganglion. The mRNA from the *CAPA* gene is present in nearly every ganglion in each post-embryonic stage. The number of cells expressing the *CAPA* gene varies during post-embryonic life, starting at 54 cells in first-instar larvae and declining to a minimum of 14 cells midway through adult development.

Key words: CAP2b, cardioacceleratory peptide, *Manduca sexta*, insect peptide, insect neuropeptide, tobacco hawkmoth, insect peptide gene, *Drosophila melanogaster*, *CAPA* gene.

Introduction

The cardioacceleratory peptides (CAPs) are a group of peptides found in insects and other invertebrate taxa. The CAPs were first isolated in the moth *Manduca sexta* as two biochemically distinct, proteinaceous peaks with cardioacceleratory bioactivity, CAP1 and CAP2 (Tublitz and Truman, 1985a–d). Further biochemical analyses determined that CAP1 was two peptides, CAP1a and CAP1b, and that the original CAP2 contained three distinct peptides, CAP2a, CAP2b and CAP2c (Tublitz et al., 1991). The first CAP to be sequenced was CAP2a, and that analysis (Cheung et al., 1992) demonstrated it was the same peptide as crustacean cardioactive peptide (CCAP; Stangier et al., 1987). CCAP performs an ever increasing variety of functions in insects and crustaceans, including increasing heart rate during wing inflation in newly emerged adult moths (Tublitz and Truman, 1985a,b), triggering the ecdysial motor program in moths and flies (Gammie and Truman, 1997; Clark et al., 2004), initiating contractions in the locust ovary (Donini and Lange, 2003) and affecting hindgut activity in caterpillars (Tublitz et al., 1992).

The second and only other member of the CAP group of peptides to have been sequenced to date is CAP2b (pELYAFPRVamide; renamed in the present paper as Mas-CAPA-1). Like CCAP, Mas-CAPA-1 is involved in numerous physiological roles in a diverse set of insects. Mas-CAPA-1 was first identified in the moth *Manduca sexta* as causing an increase in heart rate when applied *in vivo* and *in vitro* (Tublitz and Truman, 1985a–d; Tublitz et al., 1991). Mas-CAPA-1 has also been shown to stimulate the rate of fluid secretion when applied to Malpighian tubules from *Manduca* and the fruit fly *Drosophila melanogaster* (Skaer et al., 2002; Davies et al., 1995, 1997).

Mas-CAPA-1 appears to be related to several other insect peptides with CAPA-like structures. The fruit fly, *Drosophila melanogaster*, contains a deduced peptide, Drm-CAPA-1 (GANMGLYFPRVamide), with a high degree of structural

similarity, particularly at the C-terminus, to Mas-CAPA-1 (Kean et al., 2002). Although ineffective on the *Drosophila* heart (N.J.T., unpublished), this peptide is a potent activator of fluid secretion by the *Drosophila* Malpighian tubules (Kean et al., 2002). CAPA-like peptides with actions on the hyperneural muscle have been isolated from the perisympathetic organs of the cockroach *Leucophaea maderae* and the locust *Locusta migratoria* (Predel et al., 1995; Predel and Gade, 2002). There is increasing evidence that Mas-CAPA-1 and Drm-CAPA-1 are part of the CAPA family of peptides found in a variety of insects. The CAPA peptides as a group appear to be part of a larger superfamily of structurally related modulatory neuropeptides that include the periviscerokinins in insects (Wegener et al., 2002) and the small cardioactive peptides in molluscs (Whim et al., 1993).

Despite the increased interest in the CAPA peptides, there is little information about CAPA gene structure and gene expression. The release of the *Drosophila* genome sequence provided the first glimpse into the sequence of a CAPA-encoding gene (Kean et al., 2002). The aim of the present study was to further address this issue in the moth *Manduca sexta*, where much of the early work on CAPA peptides was performed. Using standard molecular methods, we have isolated and sequenced a CAPA-encoding gene in *Manduca*. We also present *in situ* hybridization data on the spatial and temporal expression pattern of this gene in all post-embryonic stages in *Manduca*.

Materials and methods

Experimental animals

Manduca sexta L. were raised at the University of Oregon as previously described (Loi and Tublitz, 1993; Loi et al., 2001). The *in situ* hybridization experiments in this paper utilized animals from every major post-embryonic stage. All stages were raised in an environmentally controlled chamber with a 17 h:7 h L:D photoperiod and a superimposed thermal period (27°C during light cycle and 25°C during dark cycle) to synchronize development. Animals were staged based on stage-specific developmental and/or behavioral events (Truman and Riddiford, 1974). D0 is designated as the day of a larval–larval molt; W0 is the day of the onset of wandering; P0 is the day of the larval–pupal molt. Other stage-specific developmental and/or behavioral markers used in this study include head capsule slippage stage (HCP; inclusive of fluid filled and airfilled head capsules) and P10 (pupal day 10, the day before the full development of the dorsal pad on the nerve cord). On the day of adult emergence, animals not yet emerged were designated as ‘pharate adults’. After emergence, adults were designated as adult day 0 (A0).

Polymerase chain reaction (PCR) screening for a CAPA-*containing transcript from a* Manduca sexta *cDNA library*

All oligonucleotides used in the PCRs described in this paper were made by Gibco (Life Technologies Inc., Grand Island, NY, USA). Taq polymerase and buffer were purchased from Clontech (Titanium advantaq; BD Biosciences, Clontech, Palo Alto, CA, USA), and dNTPs were purchased from Promega (Madison, WI, USA). The following were used for each 15 µl PCR sample: 0.3 µl of 10 $mmol^{-1}$ dNTPs, 1.5 µl of PCR buffer, 0.3 µl of Taq polymerase, 0.3 µl of each primer (concentration, 10 $pmol\ \mu l^{-1}$) and sufficient sterile water to bring the reaction volume to 15 µl. PCRs were performed in a Peltier PTC-200Thermocycler (MJ Research Inc., Incline Village, NV, USA) using program settings described below. *Manduca sexta* 8 Zap cDNA and genomic DNA were made from whole nerve cords of day 3 fifth-instar larvae by Stratagene Corporation (La Jolla, CA, USA) and re-amplified as necessary.

PCRs

As a first step in obtaining the *CAPA* gene sequence, two sets of PCRs were performed with a degenerate oligonucleotide and a universal primer using re-amplified *Manduca* cDNA from fifth-instar nerve cords as the DNA template. The first set of PCR was performed with a degenerate primer designed using the amino acid sequence of *Manduca sexta* CAPA-1. The degenerate primer was constructed to recognize the sense strand of a putative CAPA-encoding gene and had a sequence of A/GC/TTITAC/TGCITT-C/TCC(G/A/T/C)A/CG (see Fig. 1; bp 203–221). The universal primer was constructed to recognized the vector arm at the 5′ end of the insert and had the sequence GTAATACGACTCACTATAGGGC. The PCR conditions for the initial PCR were 2 min at 94°C, 30 s at 60°C and 1.5 min at 72°C for the initial cycle, followed by 34 cycles of 1 min at 94°C, 30 s at 60°C and 1.5 min at 72°C. The reaction was terminated by a 10 min period at 72°C and held at 4°C. The PCR product was separated by gel electrophoresis using a 5% polyacrylamide gel. DNA was visualized under ultraviolet illumination after a 5 min incubation with 15 µl of ethidium bromide in 100 ml of gel running buffer and a rinse in distilled water. The products from this initial PCR were subcloned and sequenced as described below.

The second set of PCRs was performed to determine the remaining sequence of the CAPA mRNA. The same PCR conditions and cDNA template were used as described above except for the primers. One primer was constructed using the consensus sequence obtained from the first set of PCRs. This primer recognized the antisense strand at 250–271 bp downstream of the CAPA coding region and had a sequence of CGTCTAAATGCTGTTCAGGTCGCAGG. The second primer was a universal primer that recognized the 3′ arm of the vector and had the sequence AATTAACCCTCAC-TAAAGGG. The products from this initial PCR were separated electrophoretically following the procedure described in the previous paragraph. The PCR products of interest were subcloned and sequenced as described below.

Subcloning and transformation

All PCR bands of interest were subcloned and transformed to generate DNA templates for sequencing. For subcloning

only, PCR products from the initial and second PCR runs were electrophoresed on 1% low-melt agarose gel (FMC Bioproducts, Rockland, ME, USA) in E buffer (48 g of Trizma base, 7.4 g of disodium EDTA dissolved in 600 ml of water, adjusted to pH 8 with acetic acid). The pieces of gel containing the bands of interest were individually excised, melted and cloned into a PCR2.1-TOPO vector using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) following the procedure specified by the manufacturer.

DNA sequencing

Cloned PCR products were purified using a Genemate miniprep kit (MOBIO Laboratories, Solana Beach, CA, USA). PCR was performed to confirm that the insert contained the PCR product of interest. Purified products were sequenced at the University of Oregon DNA Sequencing Facility using the vectors M13 forward and M13 reverse (Invitrogen) on a Beckman CEQ 8000 capillary sequencer (Beckman Coulter, Fullerton, CA, USA).

PCR screening for a CAPA *gene from a* Manduca sexta *genomic DNA*

PCR was performed on genomic *Manduca* DNA to determine the presence of introns within the coding region of the *CAPA* gene. The genomic library was made by Stratagene Corporation from DNA extracted from fifth-instar *Manduca* larvae. One primer recognized a region upstream of the putative start codon. The second primer was designed to recognize a region downstream of the putative stop codon. These two primers ensured that the resultant PCR product spanned the entire coding region. All procedures from PCR to subcloning and sequencing were as described above.

In situ *hybridization*

The *in situ* hybridization protocol used here was identical to that published elsewhere (Loi et al., 2001) except that the *in situ* probes recognized portions of the Mas-CAPA-1 mRNA. The probes were constructed using the DIG (genius 4) RNA labeling kit purchased from Roche Molecular Biochemicals (Indianapolis, IN, USA). Two probes were used; one (~366 bp) recognized the beginning of the mRNA to ~306 bp into the coding region, and the other (~212 bp) recognized the portion of the *CAPA* gene from ~390 bp after the start codon to ~158 bp after the stop codon. Because the results from the two probes were identical and because the cells expressing the transcripts corresponded to cells labeled with a Mas-CAPA-1 antibody (N.J.T., unpublished results), negative controls using a sense probe were not performed.

Results

CAPA *gene isolation and sequence in* Manduca sexta

The complete sequence of the *Manduca sexta CAPA* gene is shown in Fig. 1. The gene is 958 nucleotides long with 29 untranslated nucleotides from the beginning of the sequence to the putative start initiation site. The *CAPA* gene has a single open reading frame, which is 441 nucleotides long. The open reading frame codes for a predicted

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                                gaattcggca cgagcggaca caaaggaacc          0

M   Q   S   A   V   R   L   V   V   C   L   F   L   L   S
ATG CAG TCA GCA GTA AGG CTC GTC GTG TGC TTG TTT CTT CTC TCC      45

S   V   L   G   G   S   Y   Q   S   G   P   K   L   R   R
TCG GTT TTG GGC GGC TCG TAC CAG AGC GGC CCC AAG CTA CGT CGT      90

D   G   V   L   N   L   Y   P   F   P   R   V   G   R   A
GAT GGT GTT CTC AAC CTG TAT CCA TTC CCG CGA GTC GGC CGA GCC     135

S   H   H   T   W   Q   I   P   I   N   D   L   Y   L   E
TCT CAT CAC ACG TGG CAG ATA CCC ATC AAT GAT CTG TAT TTA GAA     180

Y   D   P   V   D   K   R   Q   L   Y   A   F   P   R   V
TAT GAT CCA GTG GAC AAG CGG CAA CTG TAC GCG TTC CCC CGG GTC     225

G   R   S   E   L   S   L   L   R   P   E   Q   H   L   D
GGA CGG AGC GAG CTC TCT CTC CTG CGA CCT GAA CAG CAT TTA GAC     270

A   L   Q   P   V   P   A   R   R   T   E   G   P   G   M
GCC CTG CAG CCA GTT CCT GCT CGG CGC ACA GAA GGT CCG GGC ATG     315

W   F   G   P   R   L   G   R   S   F   K   S   D   E   D
TGG TTC GGA CCC CGG CTC GGC AGG TCC TTC AAA AGC GAC GAA GAC     360

E   I   T   I   Q   N   N   N   L   E   R   S   E   P   E
GAA ATC ACA ATC CAA AAC AAC AAC TTG GAG CGC AGC GAG CCC GAG     405

L   M   E   R   K   K   R   N   A   H   L   N   STOP
CTT ATG GAG AGA AAG AAA CGA AAC GCG CAC TTA AAC TAA             444

tactgacaaa cagaatataa ataaagctgc aatactcgct tcgttctcac         494

ttcattataa ataacattag aatacacaga agactataga atgaatgaaa         544

tgaaaacaga ctattattcc taatgttgta ctattattaa acttttgaaa         594

atcgaaaata acgttattta tgccgaaggt ctgactttcc ctcccatatt         644

cctttccgaa aatagacaat gcacccacca tctggctaga ggtgaaaagg         694

ggcaatccaa catataccta ttattaccca aatagatttt aaatacgttg         744

aatacatgtt aattattata aggtttgttg ccaaaattat ttgtgaggct         794

gggcggtttt gttcatacga aaatgttaat aattccgatg aataattttg         844

gcaaacgttt aaatgttaat agttgtttgt aaatgcaaca atttatttaa         894

ataaagaaaa tcactcttaa aaaaaaaaaa aaaaa                        929
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Fig. 1. Complete sequence of the *Manduca sexta CAPA* (cardioacceleratory peptide 2b; Mas-CAPA-1) gene. Nucleotide and predicted peptide sequences are listed for the coding region, which starts at nucleotide 1. Orange, Mas-CAPA-2 peptide encoding region; purple, Mas-CAPA-1 encoding region; blue, Mas-PK-1 peptide encoding region; green, glycine used for C-terminal amidation; red, dibasic post-translational cleavage sites; STOP, stop codon.

precursor protein of 147 amino acids and contains a highly predicted signal peptide sequence with a deduced cleavage site between residues 19 and 20 (SignalP V1.1; Nielsen et al., 1997). The predicted prepropeptide encodes a single copy of each of three deduced propeptides: a Mas-CAPA-1 propeptide, with a Q substituted for an E at the N-terminus (QLYAFPRV; bases 202–225), and two novel CAPA-related propeptides [Mas-CAPA-2 (DGVLNLYPFPRV), bases 91–126, and Mas-PK-1 (TEGPGMWFGPRL), bases 298–333]. All three deduced propeptides in the predicted precursor protein are preceded by an RR or RK doublet and followed by an R singlet of basic amino acids that together serve as internal proteolytic cleavage sites during post-translational processing of the proprotein (Loh and Gainer, 1983). The presence of a glycine residue at the C-terminus of each peptide coding region immediately prior to the monobasic residue cleavage site suggests that the mature form of each deduced peptide is amidated at the C-terminus (Fig. 1). Protein sequence data for the biologically active form of CAPA (pyroELYAFPRVamide; Huesmann et al., 1995) indicate the presence of an N-terminal pyroE, which is likely to be generated from a post-translational cyclization of the N-terminal Q of the CAPA propeptide into a pyroE in the mature version. There are 485 untranslated base pairs between the stop codon and the beginning of the poly (A) tail. We did not find any indication of intron(s) in the *CAPA* gene using the same procedures on genomic DNA. The predicted molecular masses of DGVLNLYPFPRVa and TEGPGMWFGPRLa are 1387.7 and 1345.6 Da, respectively (http://www.expasy.ch/tools/peptide-mass.html; Wilkins et al., 1999). The complete sequence is accessible at NCBI's GenBank (GenBank accession #AY649544).

Spatial expression of the CAPA *gene in the* Manduca *CNS*

Two probes were used to investigate the expression of the *CAPA* gene. One recognized 366 bp of the 5′ end of the mRNA. The other was made against a 216 bp region in the 3′ non-coding region. Both probes labeled the same cells, with differences only in the intensity and background. Because the results from the two probes were identical in preliminary studies, the temporal and spatial expression data presented here were obtained with the shorter probe. The shorter probe labeled a maximum of 54 cells in the early larvae stage and only 14 cells in the pharate adult stage (Fig. 2**)**. The spatial expression of the *Manduca CAPA* gene is described in detail in the following sections.

Brain

The CAPA *in situ* probe labeled 15–16 pairs of cells in the brain of 1st- and 2nd-instar caterpillars. Of these, 7–8 pairs of cells are located dorsally, five pairs are situated ventrally and four pairs are found near the middle (Figs 2, 3, 4A–C**)**. The number of cells expressing the *CAPA* gene declines as the animal matures (Figs 3, 4). By early 5th instar, there are five pairs of *CAPA*-expressing cells, and this number increases to ~12 pairs of cells by wandering day 1 (W1). The number of CAPA cells again declines after W1 and, by pharate adult, only one pair is left (Fig. 4D). Due to the reorganization of the brain during metamorphosis, it was impossible to determine if the pair expressing the *CAPA* gene in the pharate adult brain bears any relationship to any of the *in situ* positive cells in the larval brain.

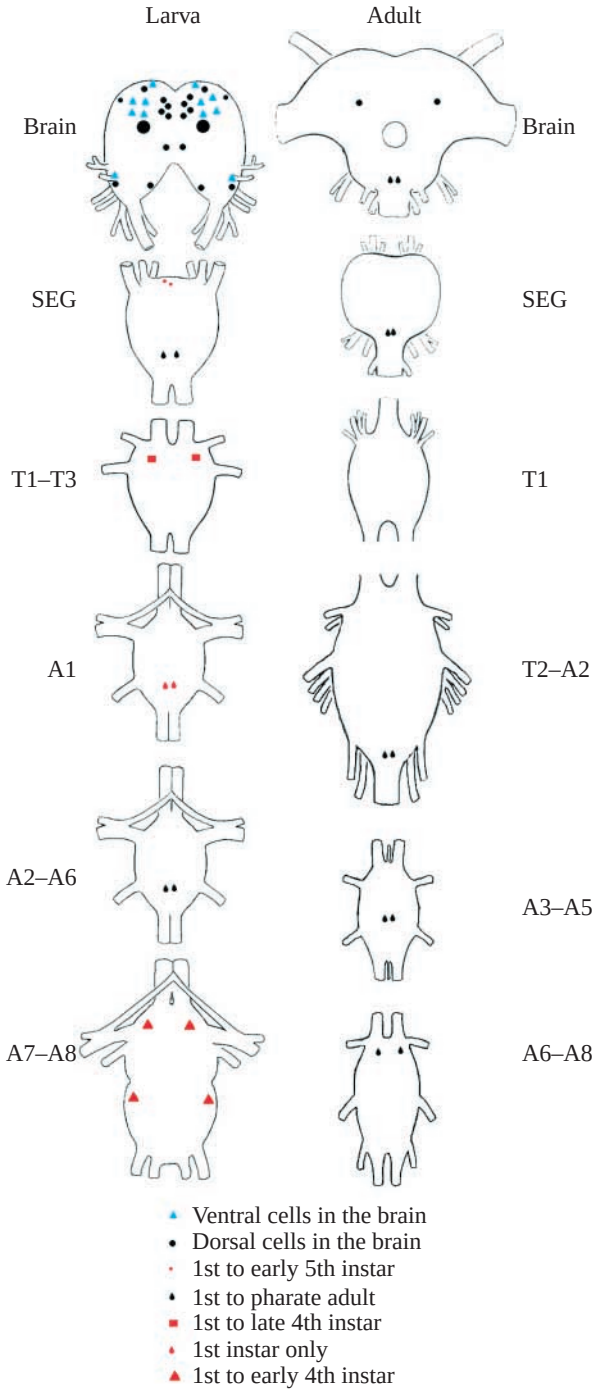

Fig. 2. Summary of all cells in the larval and adult *Manduca sexta* central nervous system (CNS) labeled with the *in situ* probe for the *CAPA* gene. SEG, sub-esophageal ganglion; T1–3, thoracic ganglia 1–3; A1–8, abdominal ganglia 1–8.

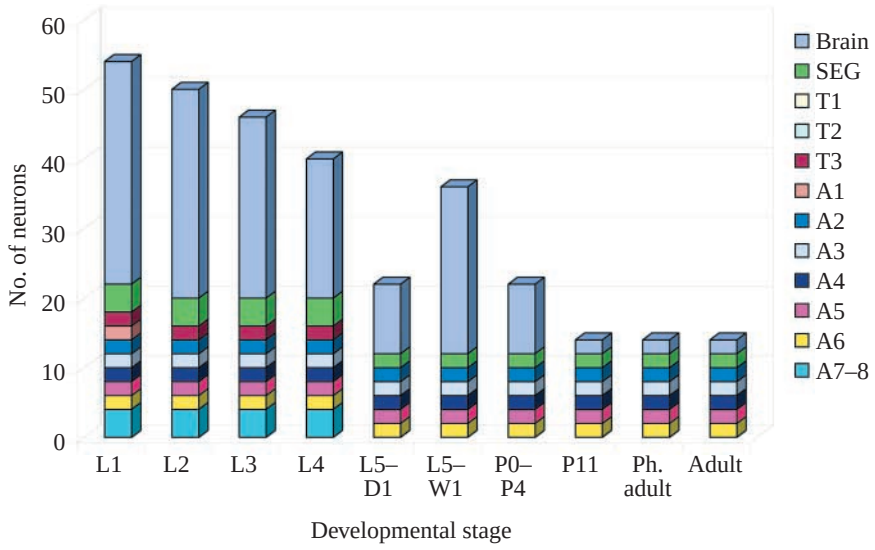

Fig. 3. Spatial and temporal expression of CAPA expressing cells in the *Manduca sexta* CNS. L, larval stages; W, wandering stage of the 5th instar larva; P, pupa; Ph, pharate; SEG, sub-esophageal ganglion; T1–3, thoracic ganglia 1–3; A1–8, abdominal ganglia 1–8.

Sub-esophageal ganglion (SEG)

There are two pairs of *CAPA*-expressing cells in the SEG. Both pairs of cells are located along the midline; one pair is located anteriorly and is substantially smaller than the other pair, which is located posteriorly (Fig. 5). The anterior pair stops expressing the *CAPA* transcript message after molting into the 5th instar. The posterior pair continues to express the *CAPA* gene into the pharate adult (Fig. 4D) and adult stages (adult day 1; not shown).

Thoracic ganglia

Expression of the *CAPA* gene transcript was not observed in the 1st and 2nd thoracic ganglia of any post-embryonic stage in *Manduca*. It is expressed in a pair of lateral cells in the 3rd thoracic ganglion (Fig. 6) in 1st-, 2nd- and 3rd-instar larvae. These cells stop expressing the CAPA transcript after the HCP stage in the 4th-instar larva and do not express it in any subsequent stage.

Abdominal ganglia

The 1st abdominal ganglion (A1) has a pair of posterior midline cells that label with the CAPA probe only in 1st-instar larvae.

The 2nd abdominal ganglion (A2) has a similar pair of posterior midline cells that express the *CAPA* gene from the 1st instar onwards. *CAPA* gene expression in these cells is very variable; only a small percentage (5–30%) of cells express the gene within any given stage of development (Fig. 7A,B). In addition to the high variability of *CAPA* gene expression in these cells, the *CAPA* gene is often transiently expressed. For example, the *CAPA* transcript is undetectable during the first 3 days of the 5th instar (D1–3), yet reappears at W1. The *CAPA* transcript in these cells disappears again in early pupae, only to reappear in the pharate adult stage.

The 3rd, 4th, 5th and 6th abdominal ganglia (A3–6) each contain a pair of posterior midline cells that are labeled by the CAPA probe. Transcript expression is very robust in these cells in all ganglia and remains detectable from the 1st instar until adult day 1 (Fig. 7C).

Terminal ganglion

The *CAPA* gene is expressed in a pair of anterior lateral cells in both terminal ganglion 7 and 8 (T7–8). The message is detectable only until D0 4th instar (Fig. 8A,B). At metamorphosis, the 6th abdominal ganglion fuses with the terminal ganglion, and a pair of cells expressing the CAPA transcript are present in the A6 neuromere of pharate adult terminal ganglia (Fig. 8C). These cells are likely to be the same *CAPA*-positive cells found in the A6 in larvae **(**Figs 2, 3).

Temporal expression of the CAPA *gene in the* Manduca *CNS*

Expression of the *CAPA* gene varies greatly across developmental stages in *Manduca* (Fig. 3). The maximum number of cells expressing the *CAPA* gene (54 cells) is found in 1st-instar larvae. As larvae mature and go through various molts, the number of cells expressing the *CAPA* gene declines. There is a significant increase in cells positive for the *CAPA* gene on W1 of the last larval instar (5th instar). Following pupation, the number of neurons expressing the *CAPA* gene product drops precipitously, reaching a minimum of 14 cells in day 1 adults.

Discussion

The Manduca CAPA *gene encodes three peptides*

The *Manduca CAPA* gene produces a single prepropeptide that is post-translationally processed to generate three small peptides: pELYAFPRVa (Mas-CAPA-1) and two previously undescribed peptides, DGVLNLYPFPRVa and TEGPGMWFGPRLa. Mas-CAPA-1 was originally identified as Mas-CAPA-1, one of a set of five CAPs biochemically isolated from the *Manduca* pharate adult nerve cord on the basis of their cardioexcitatory bioactivity (Tublitz et al., 1991). Of the five original CAPs (CAP2a, CAP2b/Mas-CAPA-1, CAP2c, CAP1a, CAP1b), only CAP2a, structurally identical to and now known as Mas-CCAP (crustacean cardioactive peptide; Stangier et al., 1987), and Mas-CAPA-1/CAP2b, have been sequenced (Cheung et al., 1992; Huesmann et al., 1995). As the number of CAPs has increased, their nomenclature has become increasingly cumbersome and confounding, and the discovery of two additional peptides in the *Manduca CAPA* gene adds to this confusion. To simplify

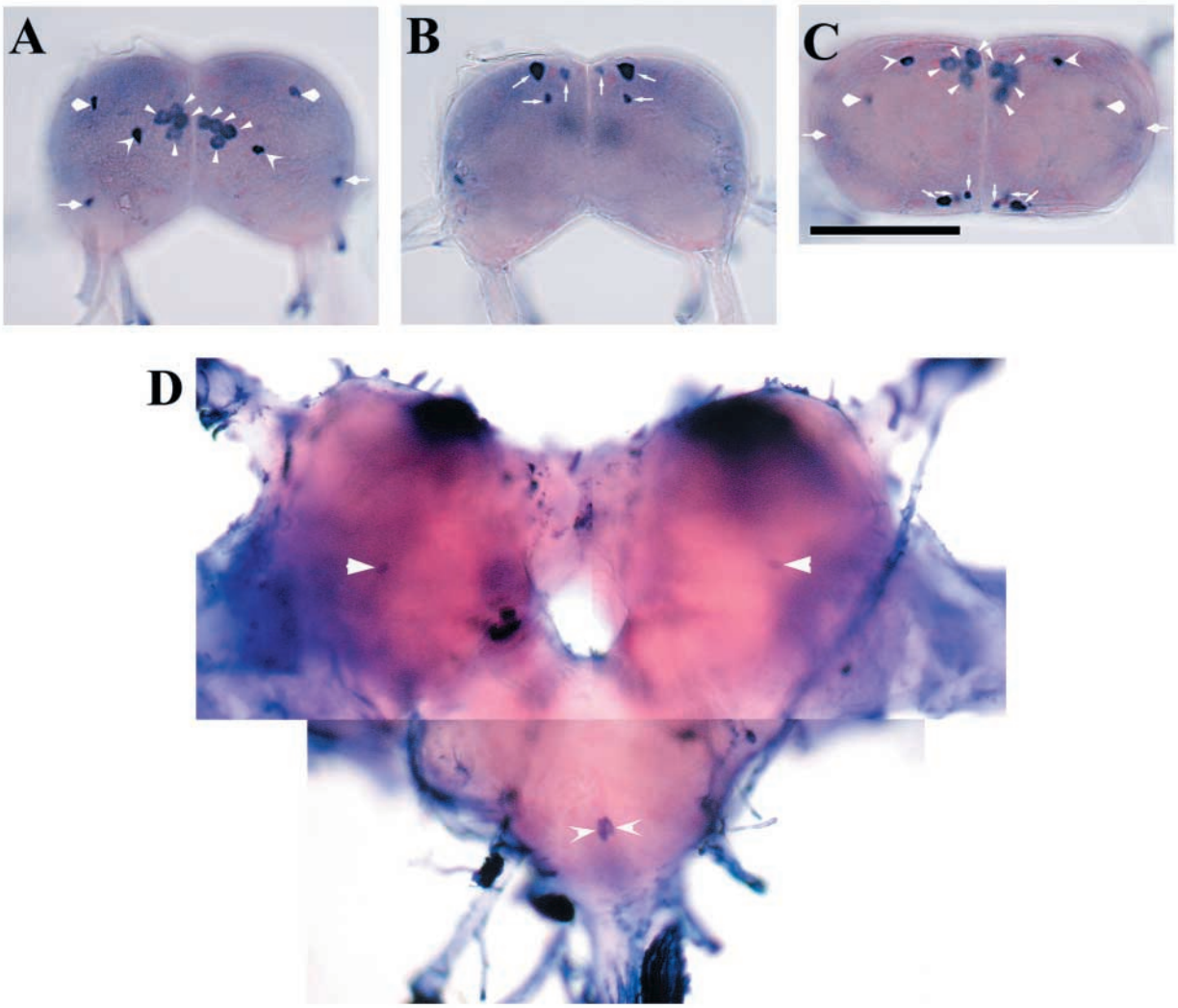

Fig. 4. CAPA transcript expression in the brain of *Manduca sexta*. Dorsal (A) and ventral (B) 3rd-instar brain. (C) Anterior view of a different 3rd-instar brain showing the 10 pairs of CAPA-expressing cells. Arrowheads, cluster of five cells near dorsal midline; concave arrowhead, single dorsal cell in the middle of each brain hemisphere; convex arrowhead, single dorsal cell located antero-laterally in each hemisphere; convex arrow, single dorsolateral cell; arrows, cluster of three ventral cells. (D, top) Anterior view of a pharate adult brain showing the CAPA-expressing cell (arrowheads); (D, bottom) anterior view of the pharate adult subesophageal ganglion fused to the brain, showing a pair of CAPA-expressing cells (concave arrowheads). Scale bar, 100 μm.

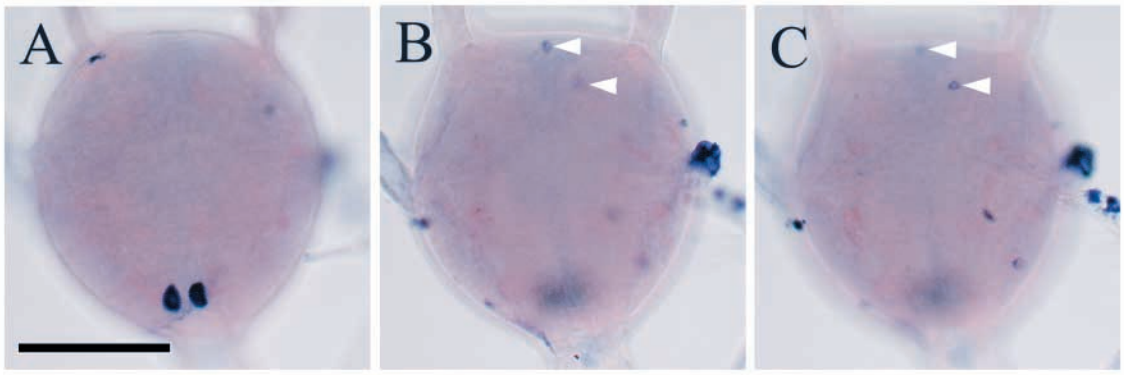

Fig. 5. CAPA transcript expression in cells in the subesophageal ganglion of a 1st-instar *Manduca sexta* larva showing the posterioventral (A) and anteriodorsal cells (B,C, arrowheads). Scale bar, 100 μm.

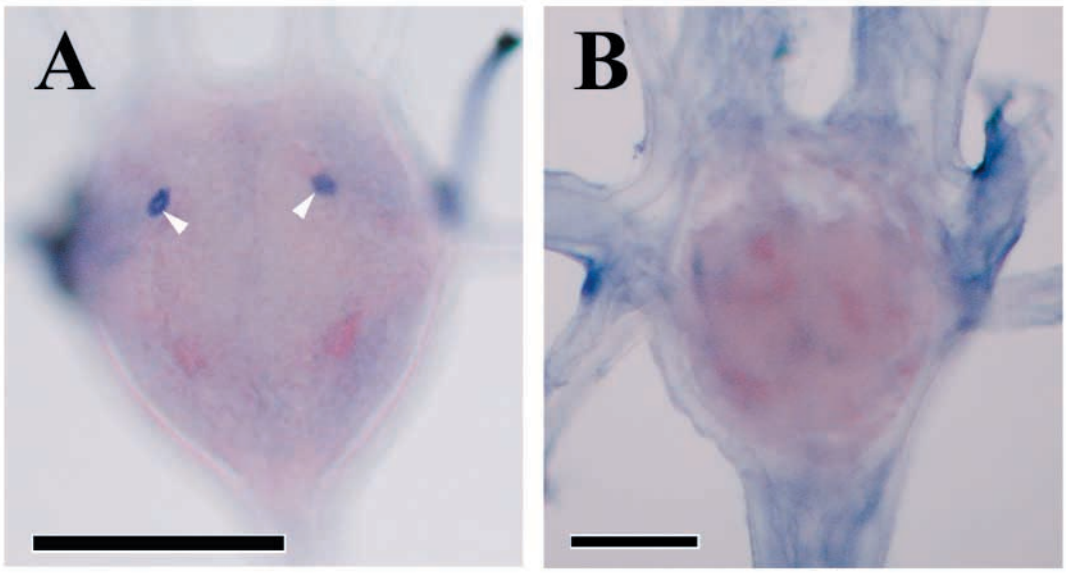

Fig. 6. CAPA transcript expression in cells in the 3rd thoracic ganglion of *Manduca sexta*. (A) 3rd thoracic ganglion from a 3rd-instar larva. (B) Absence of CAPA expression in the 3rd thoracic ganglion from a slipped-head 4th-instar larva. Scale bars, 100 μm.

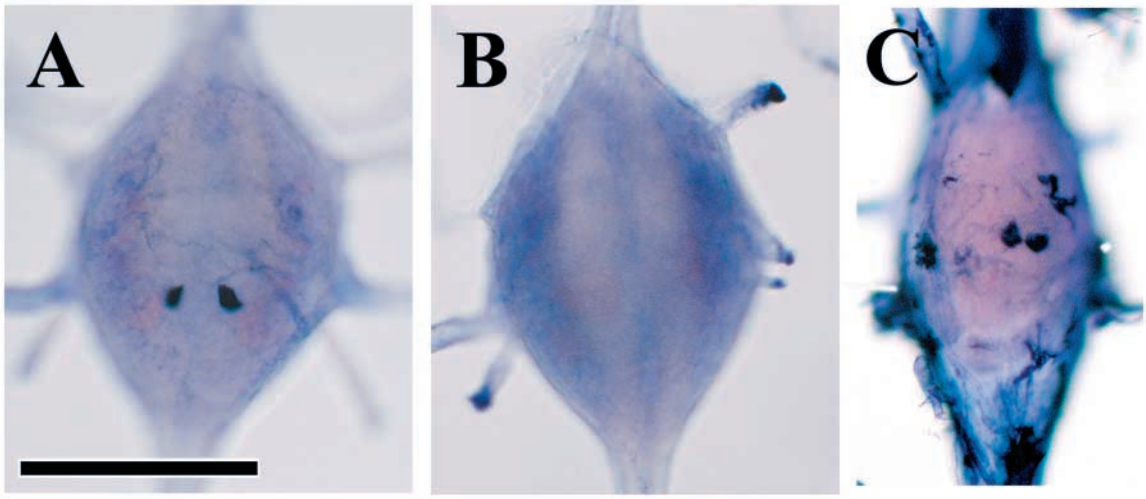

Fig. 7. CAPA transcript expression in cells in the abdominal ganglia of *Manduca sexta.* (A) *CAPA* gene expression in the 2nd abdominal ganglion (A2) of a day 1 2nd-instar larva. (B) A2 from a different day 1 2nd-instar larva with no labeled cells. (C) CAPA transcript expression in the 4th abdominal ganglion (A4) from a pharate adult. Scale bar, 100 μm.

matters and to follow the naming of peptides in the *Drosophila CAPA* gene (Kean et al., 2002), we rename pELYAFPRVa as Mas-CAPA-1 and assign the names of Mas-CAPA-2 to DGVLNLYPFPRVa and Mas-PK-1 (Pyrokinin-1) to TEGPGMWFGPRLa for reasons explained in subsequent sections.

One obvious question arising from these data is the relationship between the two new peptides (Mas-CAPA-2 and Mas-PK-1) described in the present study and the remaining three unsequenced CAPs (CAP1a, CAP1b and CAP2c). Early work on the CAPs in *Manduca* localized CAP bioactivity to the segmentally iterated perivisceral organs (PVOs; also known as the perisympathetic organs) in the *Manduca* ventral nerve cord (Tublitz and Truman, 1985a–d). Wegener et al. (2002) used MALDI-TOF mass spectral analysis to demonstrate the presence in larval *Manduca* PVOs of four proteinaceous molecules: CCAP, Mas-CAPA-1/Mas-CAPA-1 and two other unknown molecules. These two unknown molecules have monoisotopic molecular masses (1346.4 and 1388.5 Da) nearly identical to those of the two novel peptides encoded by the *Manduca CAPA* gene (Mas-CAPA-2, predicted monoisotopic mass=1388.7 Da; Mas-PK-1, predicted monoisotopic mass=1346.6 Da). These data provide direct empirical evidence supporting the hypothesis that the *Manduca* CAPA propeptide gene product is processed into three peptides, all of which are expressed in and presumably released

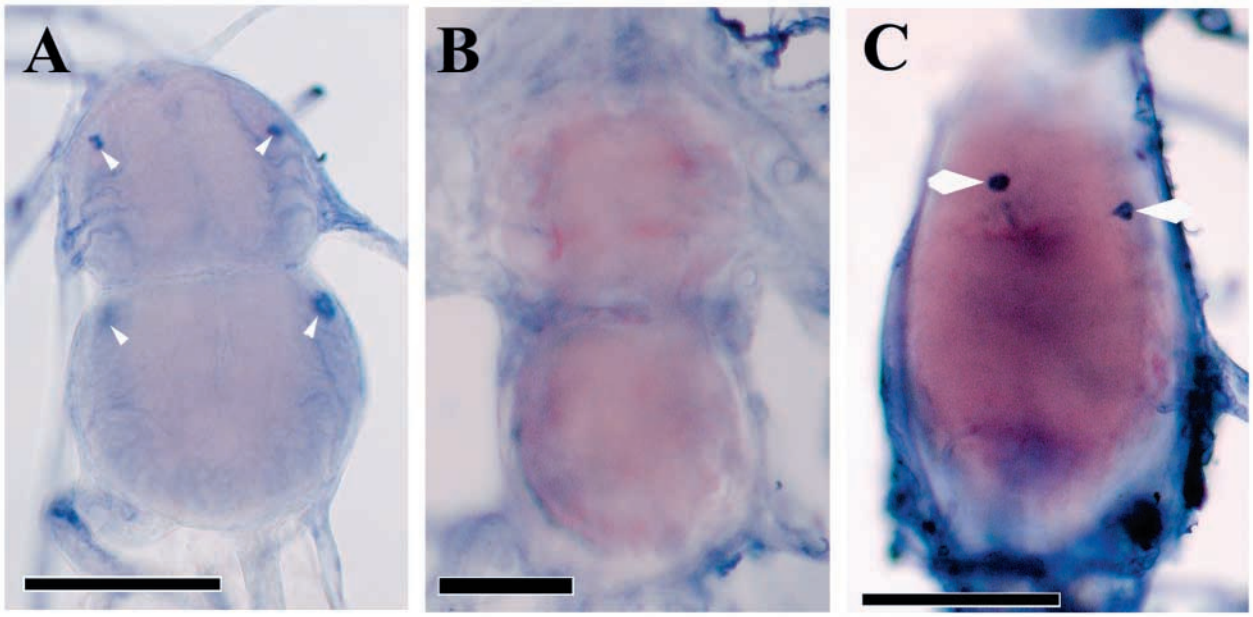

Fig. 8. CAPA transcript expression in cells in the terminal ganglia of *Manduca sexta*. (A) *CAPA* gene expressing cells in the terminal ganglion of day 1 2nd-instar larva (arrowheads). (B) Terminal ganglion of a late 4th-instar larva with no detectable staining. (C) A pharate adult terminal ganglion with two stained cells in the 6th abdominal neuromere (A6) (convex arrowheads). Scale bars, 100 μm.

from the PVOs. However, further experimentation is obviously required to determine the exact relationship, if any, between the two novel peptides on the *CAPA* gene and the CAPs.

Rationale for naming DGVLNLYPFPRVa Mas-CAPA-2

The structure of one of the deduced peptides encoded by the *CAPA* gene, DGVLNLYPFPRVa, is highly similar at the C-terminus to that of Mas-CAPA-1 (pELYAFPRVa). The *Drosophila capability* gene encodes two peptides, Drm-CAPA-1 and Drm-CAPA-2, with C-terminal sequences similar to Mas-CAPA-1 and Mas-CAPA-2 (DGVLNLYPFPRVa; Fig. 9; Kean et al., 2002), respectively. The structural homologies and similar intragenic locations of Drm-CAPA-1 and *Manduca* Mas-CAPA-1 persuaded us to rename *Manduca* Mas-CAPA-1 as Mas-CAPA-1. Using the same reasoning, and in order to remain consistent with the peptide naming system in the *Drosophila capability* gene (Kean et al., 2002), we are assigning the name of Mas-CAPA-2 to DGVLNLYPFPRVa.

Classification of Mas-CAPA-1 (pELYAFPRVa) and Mas-CAPA-2 (DGVLNLYPFPRVa)

Peptide classification has traditionally been based on structural and functional similarities. Due to the absence of detailed functional analyses for many peptides, peptide classification schemes are generally based on common elements in their primary sequences, usually at the C-terminus. C-terminal sequence similarities have been used to identify many peptide families, including the FMRFamides (Nichols et al., 1999), the small cardioactive peptides (SCPs; Perry et al., 1999) and the pyrokinins (Clynen et al., 2003). A greater challenge is presented when a novel peptide has a C-terminus with similarities to more than one peptide family. This is the case for all three deduced peptides encoded by the *Manduca CAPA* gene described in this paper. Each has a primary sequence with some similarity to the pyrokinins, the periviscerokinins (PVKs) and the SCPs (Table 1). Mas-CAPA-1 and Mas-CAPA-2 will be considered first; Mas-PK-1 will be discussed in the subsequent section.

As discussed above, Mas-CAPA-1 and Mas-CAPA-2 share highly similar C-terminal sequences, each ending in LYAXPRVa, where X is either A or P. Both are presumably amidated at the C-terminus based on the presence of a glycine at their carboxyl terminals. These two *Manduca* peptides share a high degree of structural homology with two *D. melanogaster* peptides, Drm-CAPA-1 and Drm-CAPA-2, both encoded on the *Drosophila capability* gene (Table 1; Kean et al., 2002). Although the functional significance of Mas-CAPA-2 has yet to be elucidated, it is known that Mas-CAPA-1 triggers an increase in heart rate in *Manduca* (Tublitz et al., 1991). Mas-CAPA-1 also stimulates an increase in fluid secretion when applied to isolated *Drosophila* Malpighian tubules, as do Drm-CAPA-1 and Drm-CAPA-2 (Davies et al., 1995, 1997; Kean et al., 2002). These data, taken together, clearly suggest that Mas-CAPA-1, Mas-CAPA-2, Drm-CAPA-1 and Drm-CAPA-2 should be grouped together as a family of CAPA related peptides (Table 1). The identifying structural motif for this family is the C-terminal amino acid sequence of LYAFPRVa.

The question remains, however, whether the CAPA family of peptides should be a separate peptide family or grouped within an existing peptide family. Table 1 lists the known peptide families with structural similarities to the CAPA peptides. The pyrokinins, the PVKs and the ecdysis-triggering hormones all have residues in common with the four CAPA-

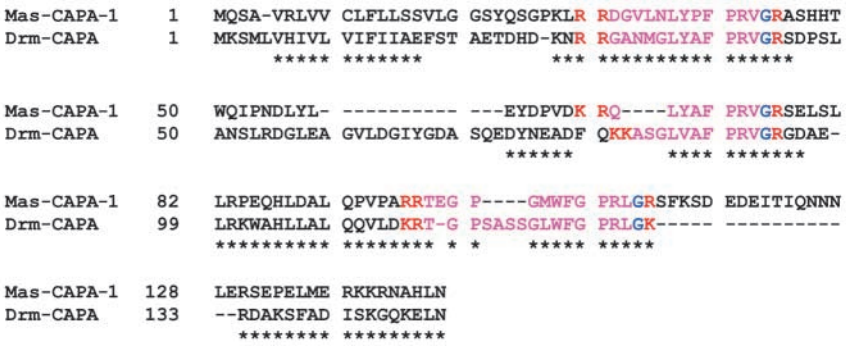

Fig. 9. Amino acid alignment between the *CAPA* gene in *Manduca sexta* and the *CAPA* gene in *Drosophila melanogaster*. Alignment derives from the DIALIGN2 protein sequence alignment program. Purple, putative peptide sequence; red, post-translated cleavage sites; blue, C-terminal glycine, presumably converted to amides; *, region of homology.

Table 1. *Sequences of CAPA (Mas-CAPA-1) peptides and their structural similarities to other insect and invertebrate peptides*

Peptide sequence	Name	Species
CAPA/Mas-CAPA-1-like peptides (**LYAFPRVa**)		
pE**LYAFPRVa**	Mas-CAPA-1 (Mas-CAPA-1)	*Manduca sexta*
GANMG**LYAFPRVa**	Drm-CAPA-1	*Drosophila melanogaster*
ASG**L**V**AFPRVa**	Drm-CAPA-2	*Drosophila melanogaster*
DGVLN**LY**P**FPRVa**	Mas-CAPA-2	*Manduca sexta*
Periviscerokinins (**GLXXXPRVa**)		
AA**GL**FQF**PRVa**	Lom-Periviscerokinin-1	*Locusta migratoria*
GSS**GL**IPFG**R**T**a**	Lem-Periviscerokinin-1	*Leucophaea maderae*
GSS**GL**ISM**PRVa**	Lem-Periviscerokinin-2	*Leucophaea maderae*
GSS**G**MIPF**PRVa**	Lem-Periviscerokinin-3	*Leucophaea maderae*
GAS**GL**IPVM**R**N**a**	Pea-Periviscerokinin-1	*Periplaneta americana*
GSS**GL**ISM**PRVa**	Pea-Periviscerokinin-2	*Periplaneta americana*
Pyrokinins (**FXPRLa**)		
HTG**F**I**PRLa**	Pea-Pyrokinin-1	*Periplaneta americana*
SPP**F**A**PRLa**	Pea-Pyrokinin-2	*Periplaneta americana*
LVP**F**R**PRLa**	Pea-Pyrokinin-3	*Periplaneta americana*
DHLPHDVYS**PRLa**	Pea-Pyrokinin-4	*Periplaneta americana*
GGGGSGETSGMW**F**G**PRLa**	Pea-Pyrokinin-5	*Periplaneta americana*
SESEVPGMW**F**G**PRLa**	Pea-Pyrokinin-6	*Periplaneta americana*
pQTS**F**T**PRLa**	Lem-Pyrokinin	*Leucophaea maderae*
pQDSGDEWPQQP**F**V**PRLa**	Lom-Pyrokinin-1	*Locusta migratoria*
LSEDMPATPADQEMYQPDPEEMESRTRY**F**S**PRLa**	Bom-PBAN-1	*Bombyx mori*
LSDDMPATPADQEMYRQDEPQIDSRTKY**F**S**PRLa**	Hea-PBAN	*Helicoverpa zea*
TDMKDESDRGAHSERGALW**F**G**PRLa**	Bom-diuretic hormone	*Bombyx mori*
NDKVDGAASGAHSERGALW**F**G**PRLa**	Hea-diuretic hormone	*Helicoverpa zea*
SVAKPQTHESLE**F**I**PRLa**	Bom-β-Neuropeptide	*Bombyx mori*
SLAYDDKSFENVE**F**T**PRLa**	Hea-β-Neuropeptide	*Helicoverpa zea*
TMS**F**S**PRLa**	Bom-γ-Neuropeptide	*Bombyx mori*
TMN**F**S**PRLa**	Hea-γ-Neuropeptide	*Helicoverpa zea*
SVP**F**K**PRLa**	hugin (short)	*Drosophila melanogaster*
TGPSASSGLW**F**G**PRLa**	CAPA-3	*Drosophila melanogaster*
TEGPGMW**F**G**PRLa**	Mas-Pyrokinin-1 (Mas-PK-1)	*Manduca sexta*
Small cardioactive peptides (molluscs) (**YLAFPRMa**)		
PG**YLAFPRMa**	SCPa	*Aplysia californica*
SQ**YLAFPRMa**	SCPa	*Lymnaea stagnalis*
MN**YLAFPRMa**	SCPb	*Aplysia californica*
QN**YLAFPRMa**	SCPb	*Lymnaea stagnalis*

Residues in bold represent family signature motifs.

like peptides in *Manduca* and *Drosophila*. Of these, the CAPA peptides have the closest structural homology with the PVKs, a group of peptides originally isolated from the cockroach *Leucophaea maderae* (Predel et al., 1995; Wegener et al., 2001) and subsequently identified in several other insect species (Predel and Gade, 2002). Early work on the PVKs raised the possibility that they required specific N-terminal and C-terminal motifs, but, with the recent identification of additional PVKs, this notion has been modified in favor of a GLXXXPRVa PVK signature motif at the C-terminus (Wegener et al., 2002). Interestingly, the primary sequences of many PVKs deviate substantially from this motif (Predel and Gade, 2002; Table 1). Wegener et al. (2002) used structural data and the similar location of PVK-immunopositive neurons in several insect species including *Manduca* to argue for the inclusion of Mas-CAPA-1 as a PVK family member. Although their evidence is consistent with their proposed classification scheme, the paucity of additional physiological and pharmacological data prevents us from agreeing, at least at present, with the proposal to include the CAPA peptides as members of the PVK peptide family. It is entirely possible that future results will support this interpretation. However, for the present, the most conservative approach, based on existing data, is to place the CAPA peptides in their own peptide family. Hence, we propose that the four CAPA related peptides (Mas-CAPA-1, Mas-CAPA-2, Drm-CAPA-1 and Drm-CAPA-2) form the basis for a separate CAPA peptide family in insects with a shared structural motif of LYAFPRVa. It should be noted that, based on structural homologies, the CAPA peptide family members are most closely related to the SCPs from molluscs (Table 1; Perry et al., 1999). However, like the PVKs, the relationship between the CAPAs and the SCPs must await further investigations.

Classification of TEGPGMWFGPRLa (Mas-PK-1)

The C-terminus of TEGPGMWFGPRLa [Mas-Pyrokinin-1 (Mas-PK-1)] matches that of the FXPRLamides, a peptide superfamily with diverse functions in insects. Widely distributed across numerous insect species, the FXPRLamides include melanization peptides (Matsumoto et al., 1992), pheromone biosynthesis activating neuropeptides (PBANs; Iglesias et al., 2002) and egg diapause induction peptides (Nachman et al., 1993). FXPRLamides also include peptides that affect visceral muscle function. These myotropic peptides, known primarily from studies on hemimetabolous insects, have been coined the pyrokinins (Zdarek et al., 2004). The pyrokinins have been localized to different neurohemal release sites in the insect CNS, including the retrocerebral complex and the PVOs (Predel et al., 1997, 1999; Predel and Gade, 2002). One of the peptides encoded by the *Drosophila capability* gene, Drm-CAPA-3 (TGPSASSGLWFGPRLa), contains the FXPRLa C-terminus motif and has been tentatively classified as a pyrokinin (Fig. 9; Table 1; Kean et al., 2002). The primary sequence of the third deduced peptide on the *Manduca* CAPA gene, TEGPGMWFGPRLa (Mas-PK-1), closely resembles that of Drm-CAPA-3 (Fig. 9; Table 1). The close homology between the *Manduca CAPA* and *Drosophila capability* genes, including the homologies between the two *Manduca* CAPA peptides and Drm-CAPA-1 and Drm-CAPA-2, suggests a similar homology between Mas-PK-1 and Drm-CAPA-3. Although the physiological significance of Mas-PK-1 is unclear, there is sufficient structural information to tentatively classify Mas-PK-1 as a member of the pyrokinin peptide family. For these reasons, we have named this peptide Mas-PK-1, using the ‘1’ designation to avoid future confusion with any as yet unidentified *Manduca* pyrokinins.

The Manduca CAPA *and the* D. melanogaster capability *genes are homologous*

The *Manduca CAPA* gene shares many characteristics with the *Drosophila capability* (*CAPA*) gene (Kean et al., 2002; Fig. 9). Both have similar length coding regions (146 vs 149 residues), and 64% of the coding region (95 residues) shows alignment between the two genes based on sequence alignment analysis using DIALIGN 2 (Morgenstern, 1999). Each gene codes for three similar peptides, all of which are probably amidated at the C-terminus based on the presence of a C-terminal glycine at the end of each peptide sequence. Each deduced *Manduca* peptide aligns closely with and shares a significant degree of sequence homology with its counterpart on the *Drosophila capability* gene (Fig. 9). Mas-CAPA-1 and the two *Drosophila CAPA* gene peptides ending in PRVa (Drm-CAPA-1 and Drm-CAPA-2) share functional properties, as each increases the rate of fluid secretion when applied to *Drosophila* Malpighian tubules (Davies et al., 1995, 1997; Kean et al., 2002). Given these functional and molecular similarities, it is likely that the *Drosophila capability* (*CAPA*) gene is the homologue of the *Manduca* gene described in this paper. We also propose that Mas-CAPA-1 and Mas-CAPA-2 are likely to be the homologous peptides in *Manduca* to the *Drosophila* peptides Drm-CAPA-1 and Drm-CAPA-2, respectively, based on similarities in their primary sequences and respective intragenic locations.

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