Dopaminergic and serotonergic innervation of cockroach salivary glands: distribution and morphology of synapses and release sites

Otto Baumann[1,*], Dana Kühnel[1,2], Petra Dames[1] and Bernd Walz[1]

[1]*Institut für Biochemie und Biologie, Zoophysiologie, Universität Potsdam, Postfach 601553, D-14415 Potsdam, Germany* and [2]*Institut für Ernährungswissenschaft, Ernährungstoxikologie, Universität Potsdam, Arthur-Scheunert-Allee 114-116, D-14558 Potsdam-Rehbrücke, Germany*

*Author for correspondence (e-mail: obaumann@rz.uni.potsdam.de)

Summary

The paired salivary glands in the cockroach are composed of acini with ion-transporting peripheral P-cells and protein-secreting central C-cells, and a duct system for the modification of the primary saliva. Secretory activity is controlled by serotonergic and dopaminergic neurons, whose axons form a dense plexus on the glands. The spatial relationship of release sites for serotonin and dopamine to the various cell types was determined by anti-synapsin immunofluorescence confocal microscopy and electron microscopy. Every C-cell apparently has only serotonergic synapses on its surface. Serotonergic and dopaminergic fibres on the acini have their release zones at a distance of ~0.5 μm from the P-cells. Nerves between acinar lobules may serve as neurohaemal organs and contain abundant dopaminergic and few serotonergic release sites. Some dopaminergic and serotonergic release sites reside in the duct epithelium, the former throughout the duct system, the latter only in segments next to acini. These findings are consistent with the view that C-cells respond exclusively to serotonin, P-cells to serotonin and dopamine, and most duct cells only to dopamine. Moreover, the data suggest that C-cells are stimulated by serotonin released close to their surface, whereas P-cells and most duct cells are exposed to serotonin/dopamine liberated at some distance.

Key words: serotonin, 5-hydroxytryptamine, dopamine, synapsin, innervation, synapse, immunocytochemistry, salivary gland, insect, cockroach, *Periplaneta americana*.

Introduction

The salivary glands in the cockroach *Periplaneta americana* are of the acinar type and can produce two different qualities of saliva, either with or without proteins (Just and Walz, 1996). The control of salivation is achieved mainly *via* serotonergic and dopaminergic neurons that originate from the suboesophageal ganglion and the stomatogastric nervous system (Ali, 1997; Baumann et al., 2002). Dopamine induces the production of saliva without proteins, whereas serotonin (5-hydroxytryptamine) leads to the exocytosis of secretory granules and the secretion of a protein-rich saliva (Just and Walz, 1996). Key mechanisms in the control of salivation, such as the identity and physiological characteristics of receptor proteins for aminergic secretagogues and the signalling cascades that mediate between activated receptors and ion transporters and the molecular machinery involved in protein secretion, respectively, are as yet poorly understood. One prerequisite for the complete comprehension of the nervous aminergic control of salivation is detailed knowledge of the spatial relationship between the release sites for serotonin and dopamine, respectively, and the various cell types engaged in saliva production and its modification.

The paired salivary glands consists of three main cell types with different functions (Fig. 1). The grape-like acini are composed of central cells (C-cells) and peripheral cells (P-cells). C-cells secrete proteins, whereas P-cells secrete ions and water into the acinar lumen (Ginsborg and House, 1980; Just and Walz, 1996). The iso-osmotic NaCl-rich primary saliva then passes through the duct system and is modified by the duct epithelial cells, resulting in the hypo-osmotic final saliva (Gupta and Hall, 1983; Lang and Walz, 2001; Rietdorf et al., 2003). Physiological studies of isolated salivary glands have further shown that these cell types differ in their sensitivity to aminergic secretagogues. C-cells are responsive only to serotonin, duct cells to dopamine, and P-cells to both serotonin and dopamine (Just and Walz, 1996; Lang and Walz, 1999a, 2001).

In order to determine whether the different responsiveness of above cell types to serotonin and dopamine is paralleled by their innervation pattern, we have examined the spatial relationship of serotonergic and dopaminergic nerve fibres to these cells. In a previous study, we began to address this question by immunofluorescence labelling of nerve fibres with anti-dopamine or anti-tyrosine hydroxylase (anti-TH), both probes for dopaminergic neurons, and with anti-serotonin

(Baumann et al., 2002). The study showed that acini are entangled in a dense plexus of dopaminergic and serotonergic fibres (Fig. 1); the former reside on the surface of the acini next to P-cells, whereas the latter invade the acini and form a dense meshwork between C-cells. Duct segments close to the acini

are locally associated with both dopaminergic and serotonergic fibres, whereas the duct segments further downstream have only dopaminergic innervation. Moreover, numerous varicosities can be detected along the nerve fibres, either in close proximity to the acinar cells or along fibre segments at a distance from the acinar tissue, indicating that serotonin and dopamine are released either as neurotransmitters at synapses or as neurohormones into the haemolymph. However, the presence of fibre varicosities can only be an indication of neurosecretion, as thickened fibre sites might also result from the accumulation of organelles.

In this study, we complement and complete our description of the serotonergic and dopaminergic innervation of the cockroach salivary gland by characterizing the release sites (synapses and varicosities). To determine the distribution of release sites by immunofluorescence confocal microscopy, we used an antibody against *Drosophila* synapsin on whole-mount preparations of salivary glands. Synapsins are actin-binding proteins that are associated with the cytoplasmic surface of synaptic vesicles and universally found at conventional chemical synapses (e.g. Valtorta et al., 1992; Südhof, 1995). Moreover, we have examined the morphology of the release sites and their spatial relationship to P- and C-cells by transmission electron microscopy.
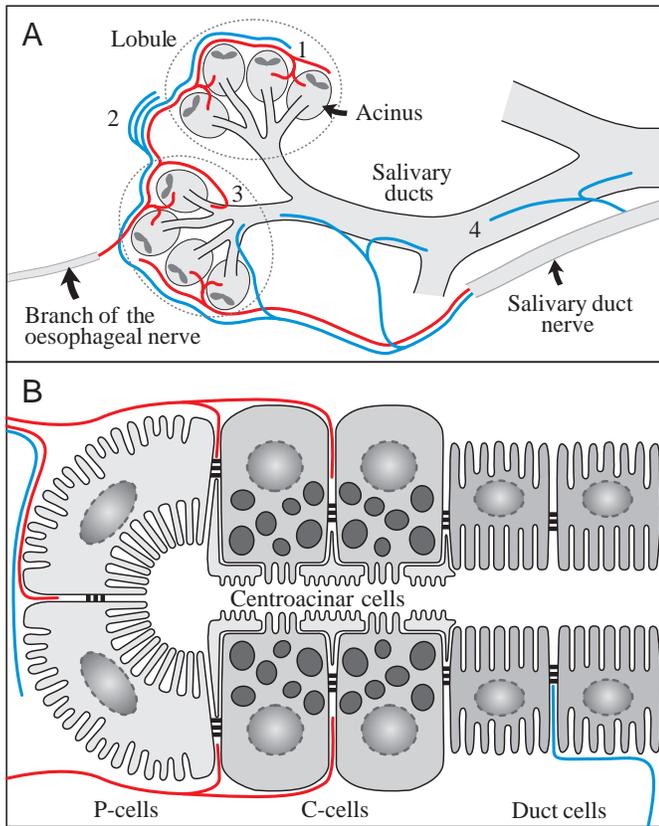

Fig. 1. Schematic representation of the organization of the cockroach salivary gland and of its serotonergic and dopaminergic innervation pattern. (A) The salivary glands are paired and consist of several lobules of secretory acini. The ducts of each gland unite to a single efferent salivary duct (4) that fuses with the opposite duct to form the main salivary duct. Innervation of the salivary gland is *via* the salivary duct nerve containing a single dopaminergic and several serotonergic axons and *via* branches of the oesophageal nerve containing serotonergic axons. Dopaminergic nerve fibres (blue) reside on the surface of the acinar tissue (1), ramify extensively in nerves that interlink adjacent acinar lobules (2) and extend to the various sections of the duct system. Serotonergic nerve fibres (red) reside on the acinar tissue (1), penetrate deeply into the acinar lobules and are also associated with sections of the duct system adjacent to the acinar tissue (3). (B) Each acinus consists of two peripheral cells (P-cells) with long microvilli and several central cells (C-cells) with numerous secretory granules. Triple black lines indicate the position of septate junctions (Just and Walz, 1994). Each acinus is covered by dopaminergic (blue) and serotonergic (red) nerve fibres. Serotonergic nerve fibres (red) extend deep into the acini between the C-cells. The apical surface of the C-cells is covered by a sheath of flattened fenestrated centroacinar cells. The duct cells have basal and apical infoldings and form a simple tubule. Dopaminergic nerve fibres (blue) extend into the epithelial layer of duct cells.

Materials and methods

Animals and preparation

A colony of the cockroach *Periplaneta americana* L. was maintained at 25°C under a 12 h:12 h light:dark regime and with free access to food and water. Young male and female imagines were killed and their salivary glands were dissected under physiological saline (160 mmol l^{-1} NaCl, 10 mmol l^{-1} KCl, 2 mmol l^{-1} CaCl$_2$, 2 mmol l^{-1} MgCl$_2$, 10 mmol l^{-1} glucose, 10 mmol l^{-1} Tris, pH 7.4), as described previously (Just and Walz, 1994).

Antibodies

Monoclonal mouse antibody SYNORF1 against *Drosophila* synapsin (Klagges et al., 1996) was provided by Erich Buchner (Universität Würzburg, Germany). Rabbit anti-serotonin and mouse anti-tubulin were obtained from Sigma (Taufkirchen, Germany). Rabbit antiserum against rat tyrosine hydroxylase (TH) was purchased from Chemicon (Temecula, CA, USA). Rabbit antiserum against *Drosophila* α-spectrin was a gift from Daniel Branton (Harvard University, Cambridge, MA, USA). Secondary antibodies conjugated to Cy3 or Cy5 were obtained from Rockland (Gilbertsville, PA, USA) and Dianova (Hamburg, Germany).

Labelling specificity has been demonstrated previously for antibodies against serotonin and TH (Baumann et al., 2002). Anti-tubulin and anti-*Drosophila* α-spectrin are known to cross-react with their corresponding antigens in various insects (Baumann and Lautenschläger, 1994; Baumann, 1997, 1998) and also identify a single protein of the appropriate molecular weight on western blots of cockroach tissues (data not shown).

Immunofluorescence labelling

Salivary glands were fixed and processed for immunofluorescence labelling as described previously (Baumann et al., 2002). Anti-synapsin was applied at a dilution of 1:25 (v/v), anti-serotonin at 1:10 000, and anti-TH at 1:200. In order to locate the various acinar cells and to provide a spatial reference for the position of the immunoreactive structures, specimens were colabelled with BODIPY-FL phallacidin (Molecular Probes, Eugene, OR, USA), a phallotoxin that binds specifically to F-actin. Fluorescence images were recorded using a Zeiss LSM 510 confocal microscope (Carl Zeiss, Jena, Germany) equipped with a 488 nm argon laser, a 543 nm helium–neon laser and a 633 nm helium–neon laser.

Quantitative analysis of colocalization

Cryostat sections of salivary glands or entire glands were colabelled with anti-synapsin and either anti-serotonin or anti-TH. By using the software MetaMorph (Universal Imaging Corp., Downingtown, PA, USA), confocal fluorescence images of the specimens were thresholded and binarized. In order to obtain the number of putative release sites for neurotransmitters and/or neurohormones, structures <10 pixels (1 pixel=0.11 μm×0.11 μm) and >100 pixels were eliminated within the anti-synapsin image, and the total number of immunoreactive structures was counted; structures <10 pixels (<0.12 μm^2) were smaller than the limit of optical resolution of the microscope and may have resulted from image noise or from synapsin-positive foci out of focus, and structures >100 pixels (>1.2 μm^2) represented homogeneously labelled axons or autofluorescent tracheoles. Although we did not place any constraints on the shape of structures selected by these procedures, comparison of the resulting images with the original fluorescence images confirmed that all and only synapsin-positive foci were selected by this method. Costained structures were then identified by multiplication of each pixel value in the binarized anti-synapsin image with the value of the corresponding pixel in the binarized anti-serotonin or anti-TH image. This procedure resulted in the elimination of structures that exhibited fluorescence in a single channel (pixel value in channel 1× value of the corresponding pixel in channel 2: 1×0=0) whereas structures that exhibited fluorescence in both channels (pixel value in channel 1× pixel value in channel 2: 1×1=1) were present in the resulting image. The number of colabelled structure was then counted by MetaMorph.

Biochemical methods

For western blot analysis, specimens (cockroach brains, cockroach salivary glands, *Drosophila* heads) were homogenized on ice in reducing sample buffer (Carl Roth, Karlsruhe, Germany). The preparations were boiled for 5 min and then centrifuged for 10 min at 16 000 g to remove non-solubilized material. Samples were loaded on 12% sodium dodecyl sulphate (SDS)-polyacrylamide gels, electrophoresed, and transferred onto nitrocellulose sheets. Western blotting was then carried out as described in detail previously

(Baumann, 2001), by using anti-synapsin at a dilution of 1:100 (v/v) and enhanced chemiluminescence for antibody detection.

Cosedimentation assays with F-actin were performed with proteins isolated from nervous tissue of the cockroach. Two brains were dissected and homogenized in extraction buffer containing 25 mmol l^{-1} KCl, 10 mmol l^{-1} Tris, 1 mmol l^{-1} EGTA, 5 mmol l^{-1} MgCl$_2$, 1 mmol l^{-1} DTT, 0.1 mol l^{-1} sucrose, 0.5% Triton X-100 (pH 7.4), supplemented with a protease inhibitor cocktail (Sigma). The preparation was centrifuged at 200 000 g for 30 min at 4°C and the supernatant was incubated for 1 h on ice with 6.5 μg ml^{-1} phalloidin-stabilized F-actin, purified from rabbit muscle (Pardee and Spudich, 1982). Actin filaments and bound proteins were sedimented by centrifugation at 200 000 g and the pellet and the supernatant were processed for western blot analysis as described above.

Electron microscopy

Salivary glands were isolated, fixed and embedded in Epon as described in detail previously (Just and Walz, 1994). Ultrathin sections were treated with uranyl acetate and lead citrate and viewed in a CM100 electron microscope (Philips, Eindhofen, The Netherlands) operated at 80 kV.

Results

*Anti-*Drosophila-*synapsin as a probe for synapses and release sites*

Monoclonal antibody SYNORF1 against *Drosophila* synapsin has been used previously as a probe for synapses in various arthropods (e.g. Fabian-Fine et al., 1999a,b; Skiebe and Ganeshina, 2000). To examine its cross-reactivity with cockroach synapsin, the antibody was applied to western blots of cockroach brains and salivary glands (Fig. 2A). For both tissues, anti-synapsin labelled a broad band that comigrated with *Drosophila* synapsin at about 80 kDa. In *Drosophila*, this band actually consists of a triplet of 70, 74 and 80 kDa (Klagges et al., 1996) but this was not resolved on our gels. A fourth isoform of synapsin has been characterized previously in *Drosophila* nervous tissue and was also detected as a faint band at about 140 kDa on our western blots. In cockroach brain and salivary gland tissue, however, there was no further anti-synapsin-positive band in this molecular mass range.

Synapsins have the ability to bind to filamentous actin (Benfenati et al., 1992; Valtorta et al., 1992). To examine whether the anti-synapsin-positive (ASP) cockroach polypeptides also displayed this property, we incubated an extract of brain tissue with actin filaments, pelleted the actin filaments, and probed the pellet and the supernatant fraction for the presence of ASP proteins. As shown in Fig. 2B, the ASP proteins copelleted with F-actin, similar to the F-actin-binding protein α-spectrin. Tubulin, used as a negative control, remained entirely in the supernatant, demonstrating the specificity of the assay.

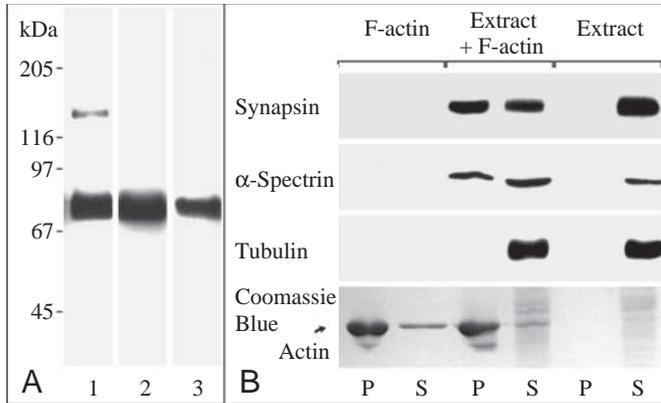Provided that the immunoreactive cockroach proteins are synapsins, they would be expected to be enriched within

Fig. 2. Cross-reactivity of anti-*Drosophila*-synapsin with cockroach synapsin. (A) Western blot analysis of *Drosophila* head (lane 1), cockroach brain (lane 2) and cockroach salivary gland (lane 3) with anti-synapsin. A broad band with an electrophoretic mobility of about 80 kDa is intensely labelled in all preparations. In *Drosophila*, an additional synapsin isoform is detected at about 140 kDa. (B) *In vitro* binding of the synapsin-positive cockroach protein to actin filaments. An extract of cockroach brain was incubated with F-actin; actin filaments were then pelleted by high-speed centrifugation. In the absence of F-actin, the anti-synapsin-positive proteins remain entirely in the supernatant (S), whereas in the presence of F-actin, a substantial fraction of the anti-synapsin-positive proteins is detected in the pellet (P) together with F-actin. The actin-binding protein α-spectrin provides a positive control, and tubulin a negative control.

neurons at presynaptic sites. Fig. 3 demonstrates that this is the case. In serotonergic nerve fibres that innervate the reservoir muscle (Baumann et al., 2002), anti-synapsin immunoreactivity was highly concentrated at varicosities and in short processes extending laterally from the axons, whereas the axons proper displayed only faint immunoreactivity (Fig. 3A–C). Within these varicosities, anti-synapsin immunoreactivity was not evenly distributed but was enriched at one side, possible the region around the active zone (Fig. 3C). To examine whether anti-synapsin

immunoreactivity is not only concentrated at conventional synapses but also at neurohaemal release sites, we colabelled salivary nerves with anti-synapsin and anti-serotonin (Fig. 3D–F). In addition to two thick, centrally localized, non-serotonergic axons, each salivary nerve contains several thin serotonergic nerve fibres that form a neurohaemal structure on the surface of the nerve (Davis, 1985; Ali 1997; Baumann et al., 2002). Here, intense immunoreactivity for anti-synapsin was concentrated within spine-like structures that extended from the serotonergic fibres towards the surface of the salivary nerve. Anti-synapsin immunoreactivity was faint within the serotonergic axons proper but was relatively intense and homogeneous within the two thick axons at the nerve centre. We thus conclude that anti-*Drosophila*-synapsin crossreacts with cockroach synapsins and that foci of intense anti-synapsin immunoreactivity (=ASP foci) represent release sites for neurotransmitters or neurohormones.

Distribution of anti-synapsin immunoreactivity over and within the salivary gland

Using anti-synapsin, we analysed the distribution of putative release sites for neurotransmitters and/or neurohormones in whole-mount preparations of salivary glands (Fig. 4). ASP foci were detected throughout the various parts of the salivary gland complex, *viz.* the acinar tissue, the nerves interconnecting the acinar lobules and the salivary ducts. The alignment of ASP structures in rows suggested that they were distributed along the length of individual axons.

In acinar lobules, numerous ASP foci were detected both on the surface of and deep within the acinar tissue (Fig. 4A–F). Every pair of P-cells, visualized by a bow-tie-like staining pattern with phallotoxin, had at least one row of ASP foci at its periphery (Fig. 4B,C). In some cases, P-cell pairs were almost completely surrounded by ASP structures. Numerous rows of ASP foci also extended deep into the acinar tissue, residing among C-cells. In nerves that interconnected adjacent acinar lobules, individual axons were stained for synapsin homogeneously over their entire length (Fig. 4G). In addition,
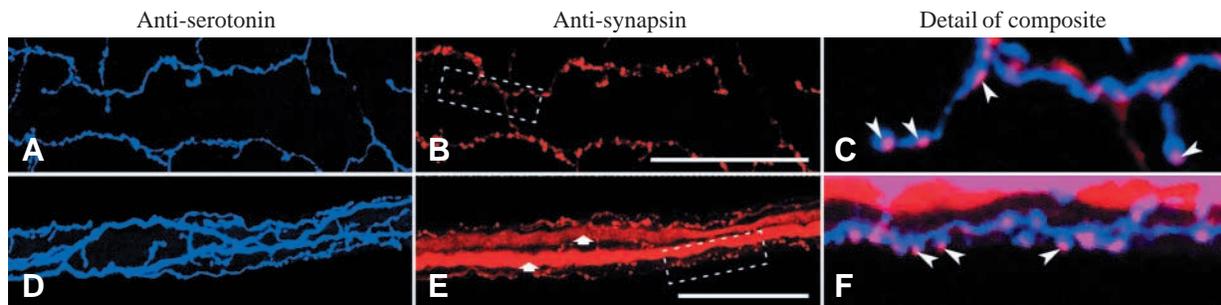

Fig. 3. Immunolocalization of synapsin in serotonergic nerve fibres at the release sites for neurotransmitters or neurohormones. (A–C) Nerve fibres in a cockroach muscle double-stained with anti-serotonin and anti-synapsin. Anti-synapsin immunoreactivity is highly concentrated at the sides of the nerve terminals (arrowheads in C). (D–F) A salivary duct nerve costained with anti-serotonin and anti-synapsin. The salivary duct nerve contains two thick, centrally localized axons that are non-serotonergic and homogeneously stained with anti-synapsin (E, arrows). These axons are surrounded by several thin serotonergic axons that ramify and have numerous short sidebranches forming a neurohaemal organ. Anti-synapsin is highly concentrated at the distal ends of these sidebranches (arrowheads in F). Bars, 50 μm.

the nerves contained numerous foci of intense ASP immunoreactivity. Generally, homogenously stained axons had a central position, whereas ASP foci resided close to the surface of the nerves. Along the salivary duct system, ASP structures were sparse (Fig. 4H–K). Rows of ASP structures resided on the outer surface of the duct epithelium or they extended deep into the epithelial layer, being localized close to the apical surface of the epithelial cells.

In summary, these results suggest that numerous release sites for neurotransmitters and/or neurohormones are
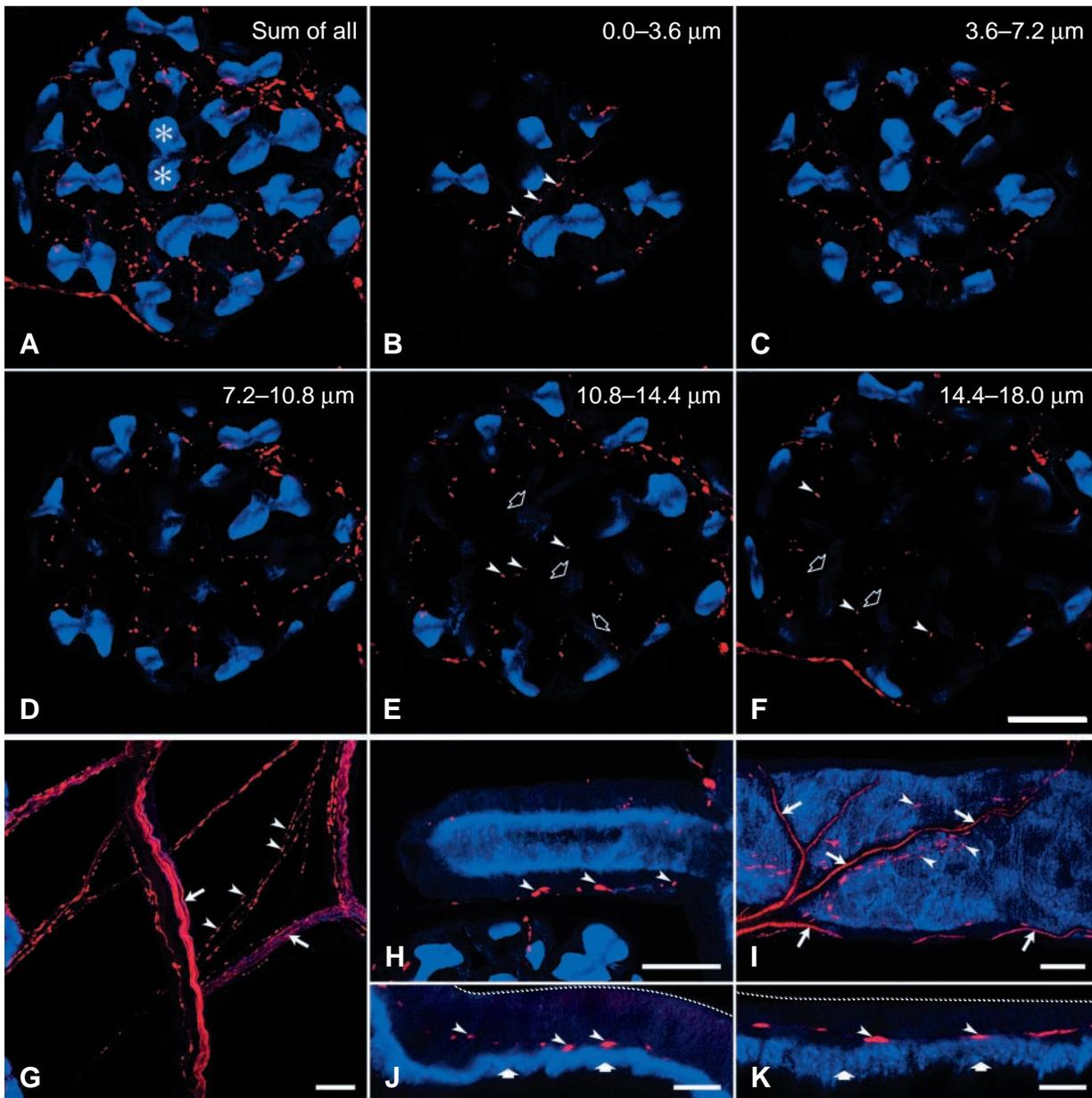

Fig. 4. Distribution of anti-synapsin-positive (ASP) structures within the salivary gland. Whole-mount preparations of salivary glands were costained with anti-synapsin (red) and BODIPY-FL phallacidin (blue) and imaged by confocal microscopy. (A–F) A series of confocal sections through an acinar lobule. Each image shows the sum of 8 consecutive optical sections (inter-section distance: 0.45 µm) representing a total thickness of 3.6 µm. (A) The sum of all confocal images. The P-cells are arranged in pairs and their apical arrays of phallotoxin-stained microvilli appear as 'bow ties' (asterisks). The C-cells are identified by short phallotoxin-labelled microvilli (E,F, open arrows) on their luminal surface. Rows of ASP foci (arrowheads) reside on the surface of the acinar tissue, next to P-cells (B), and extend deep into the acinar tissue, residing amongst C-cells (E,F). (G) Nerves interconnecting adjacent acinar lobules contain rows of ASP foci (arrowheads) and axons with homogeneous labelling for synapsin (arrows). (H,I) A small salivary duct (H) and a large efferent duct (I) with ASP foci (arrowheads) on the surface of the epithelial layer. Arrows indicate autofluorescent tracheoles. (J,K) Horizontal sections through a small duct (J) and a large duct (K) demonstrate that the ASP foci also reside within the duct epithelium, between the apical surface covered with phallotoxin-labelled microvilli (broad arrows) and the basal surface (broken line). Scale bars, 25 µm (A–I); 10 µm (J,K).
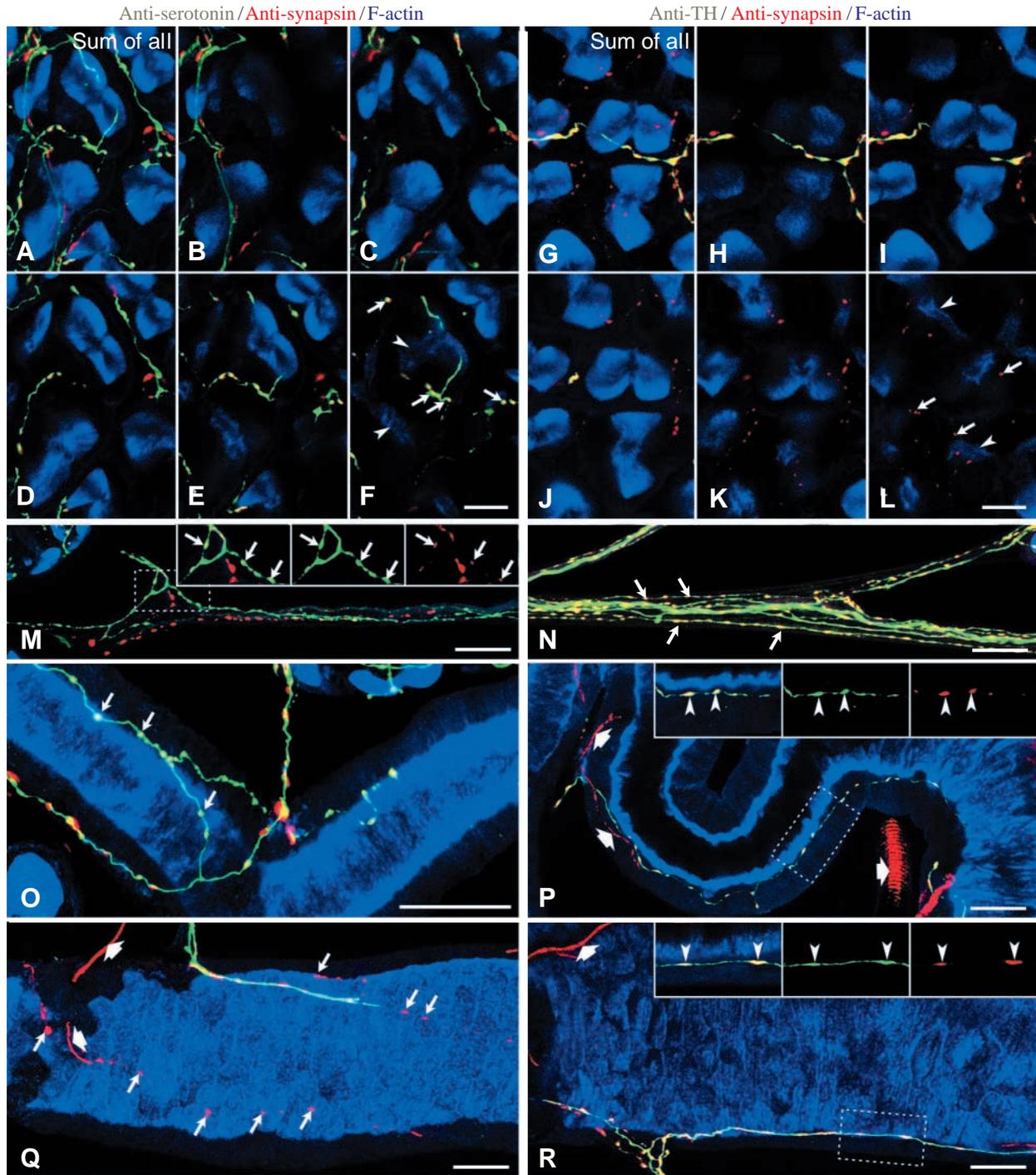
Fig. 5. Colocalization of synapsin-enriched structures with serotonergic and dopaminergic fibres. Whole-mount preparations of salivary glands were triple-labelled with anti-synapsin (red), BODIPY-FL phallacidin (blue), and either anti-serotonin (green) or anti-TH (green). (B–D,H–L) Series of confocal images through acinar tissue, each image representing the sum of 6 consecutive optical sections (inter-section distance: 0.4 μm). (A,G) The sum of all images. ASP foci on the outer surface of the acinar tissue colocalize with either anti-serotonin (B–D) or anti-TH (H–J). ASP foci lie deep in the acinar tissue, amongst C-cells that are identified by short phallotoxin-labelled microvilli (arrowheads) on their luminal surface, and colocalize with anti-serotonin only (arrows in F,L). (M,N) Within the nerves that interlink adjacent acinar lobules, most ASP foci (arrows) colocalize with anti-TH; few foci colocalize with anti-serotonin (insets in M). (O,P) ASP foci on initial duct segments colocalize with either anti-serotonin or anti-TH. (Q,R) On the large salivary ducts, ASP foci colocalize with anti-TH almost exclusively. (Insets in P,R) Horizontal sections through a duct at the position indicated by the dotted boxes in (P and R) demonstrate the distribution of ASP foci along dopaminergic fibres within the duct epithelium. Broad arrows indicate autofluorescent tracheoles. Scale bars, 10 μm (A–L); 25 μm (M–R).

associated with both P-cells and C-cells of the acinar tissue. Moreover, the nerves interconnecting acinar lobules may function as neurohaemal organs as they contain a large number of synapsin-enriched foci. Finally, there appears to be a low number of release sites for neurotransmitters/neurohormones along the salivary ducts, both on their outer surface and between epithelial cells.

Colocalization of synapsin-enriched structures with serotonergic and dopaminergic fibres

By triple-labelling with phallotoxin, anti-synapsin and either anti-serotonin or anti-TH, we examined whether the above-mentioned release sites were serotonergic or dopaminergic (Fig. 5). Anti-TH was used as a probe for dopaminergic neurons, because the fixation methods required for staining with anti-dopamine (see Baumann et al., 2002) interfered with the staining for synapsin.

On the outer surface of the acinar lobules, colabelling of ASP foci was observed with anti-serotonin and anti-TH (Fig. 5A–F,G–L). Dopaminergic ASP foci were sparsely distributed on the acinar tissue, whereas serotonergic ASP foci were relatively frequent and detected next to almost every pair of P-cells. ASP foci within the acinar tissue, suggesting a location between C-cells, colocalized exclusively with serotonergic nerve fibres. In nerves interconnecting acinar lobules, few ASP foci appeared to be serotonergic, whereas most foci were costained with anti-TH (Fig. 5M,N). Finally, on initial segments of the duct system, ASP foci were observed along serotonergic and dopaminergic nerve fibres (Fig. 5O,P). The segments of the duct system further downstream exhibited ASP foci exclusively along dopaminergic nerve fibres (data not shown), whereas the large salivary ducts (no. 4 in Fig. 1A) has most ASP foci along dopaminergic nerve fibres and few ASP foci along serotonergic nerve fibres (Fig. 5Q,R).

In addition to serotonergic and dopaminergic axons, the salivary nerve contains a thick axon of unknown neurotransmitter content (Ali, 1997; Baumann et al., 2002). We thus wanted to determine whether a fraction of ASP foci on or within the salivary gland neither colocalized with anti-serotonin nor anti-TH. Unfortunately, technical reasons prevented triple-labelling with anti-synapsin, anti-serotonin

and anti-TH. Morphometric analyses of specimens double-labelled with anti-synapsin and either anti-serotonin or anti-TH suggested, however, that the relative number of non-serotonergic/non-dopaminergic release sites must be small. In cryostat sections through acinar lobules, 88.8±7.4% of the ASP foci on or within the acinar tissue were serotonergic, and 11.3±7.4% of the foci were colabelled with anti-TH (Fig. 6A). In nerves interlinking adjacent acinar lobules, 22.6±19.1% of the ASP foci were serotonergic, and 74.7±14.0% of ASP foci were costained with anti-TH (Fig. 6B).

Ultrastructural characteristics of release sites

The morphology of the release sites for neurotransmitters/neurohormones was investigated by transmission electron microscopy. Structures considered to be release sites satisfied the following criteria: (1) an accumulation of synaptic vesicles, (2) the absence of a glial sheath, and (3) the presence of an active zone with an electron-dense area on the cytoplasmic face of the plasma membrane. In cases of ideal planes of sectioning, this electron-dense area was seen as a ribbon that extended 80–100 nm into the cytoplasm (Fig. 7C, insets).

In accordance with the results of anti-synapsin immunohistochemistry, release sites were observed (1) within the acini embedded between C-cells, (2) within nerves running along the surface of acinar lobules and thus residing close to P-cells, and (3) within nerves that interconnected acinar lobules. Although we also examined cross-sections through salivary ducts, we could only detect axonal profiles (data not shown) but no release sites. As the number of ASP foci in the ducts is low, release sites may be very rare and extensive serial sectioning is required to detect such structures by electron microscopy.

Release sites within the acinar tissue had a uniform appearance (Fig. 7A,B). The structures were without a basal lamina and thus separated only by a narrow extracellular space from the plasma membrane of the C-cells. These release sites can thus be classified as synapses. The synaptic profiles contained numerous small electron-lucent vesicles with a mean diameter of 53.2±6.8 nm (mean ± s.d.; $N=164$). Dense-core vesicles with a mean diameter of 88.4±14.2 nm ($N=27$) were also present at low numbers at these release sites. The clear
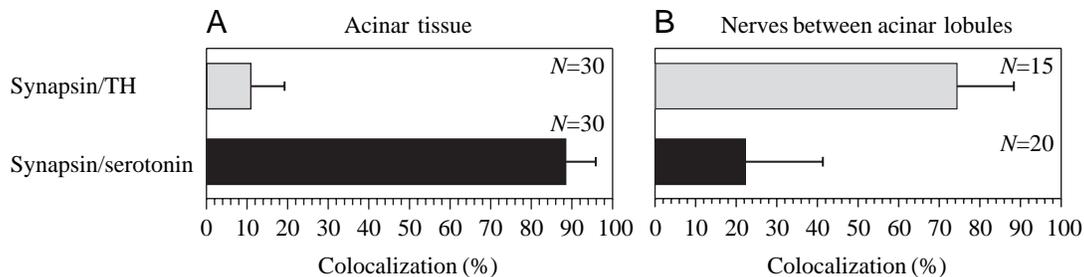
Fig. 6. Quantitative analysis of the colocalization of anti-synapsin-positive (ASP) foci with either anti-serotonin or anti-TH. (A) Most ASP foci on and within acinar tissue colocalize with anti-serotonin. (B) In neurohaemal structures between acinar lobules, the majority of ASP foci colocalizes with anti-TH, and a smaller fraction colocalizes with anti-serotonin. Values means ± s.d. (*N*=number of images analysed).

vesicles clustered around the active zones, whereas the dense-core vesicles appeared to be homogeneously distributed throughout the synaptic profiles. No 'postsynaptic' specialization could be detected on the adjacent region of the plasma membrane of the C-cells. It should be noted, however, that the cytoplasm of the C-cells was electron-dense and may have prevented the unequivocal identification of postsynaptic densities.

Release sites at the surface of nerves that run superficially on acinar lobules (Fig. 7C) or that interconnecting acinar lobules (Fig. 7D–F) were separated from the P-cell surface and the haemolymph space, respectively, by a basal lamina. These release sites can thus be regarded as neurohaemal structures. Here, at least two different types (A and B) of release sites could be distinguished by their vesicle content. Both of them

contained numerous electron-lucent vesicles of similar size (Type A: 53.7±11.4 nm, N=301; type B: 50.1±9.5 nm, N=155) that were clustered near the active zones. The size, morphology and proportion of dense-core vesicles, however, differed between the two types of release sites. Type A release sites contained few dense-core vesicles (98.9±19.5 nm; N=31). Type B release sites were observed less frequently and had numerous dense-core vesicles of a larger size (122.8±23.3 nm; N=45) and of lower electron density.

Discussion

Previous immunofluorescence studies have demonstrated that cockroach salivary glands are entangled in a dense meshwork of nerve fibres (Davis, 1985; Elia et al., 1994;
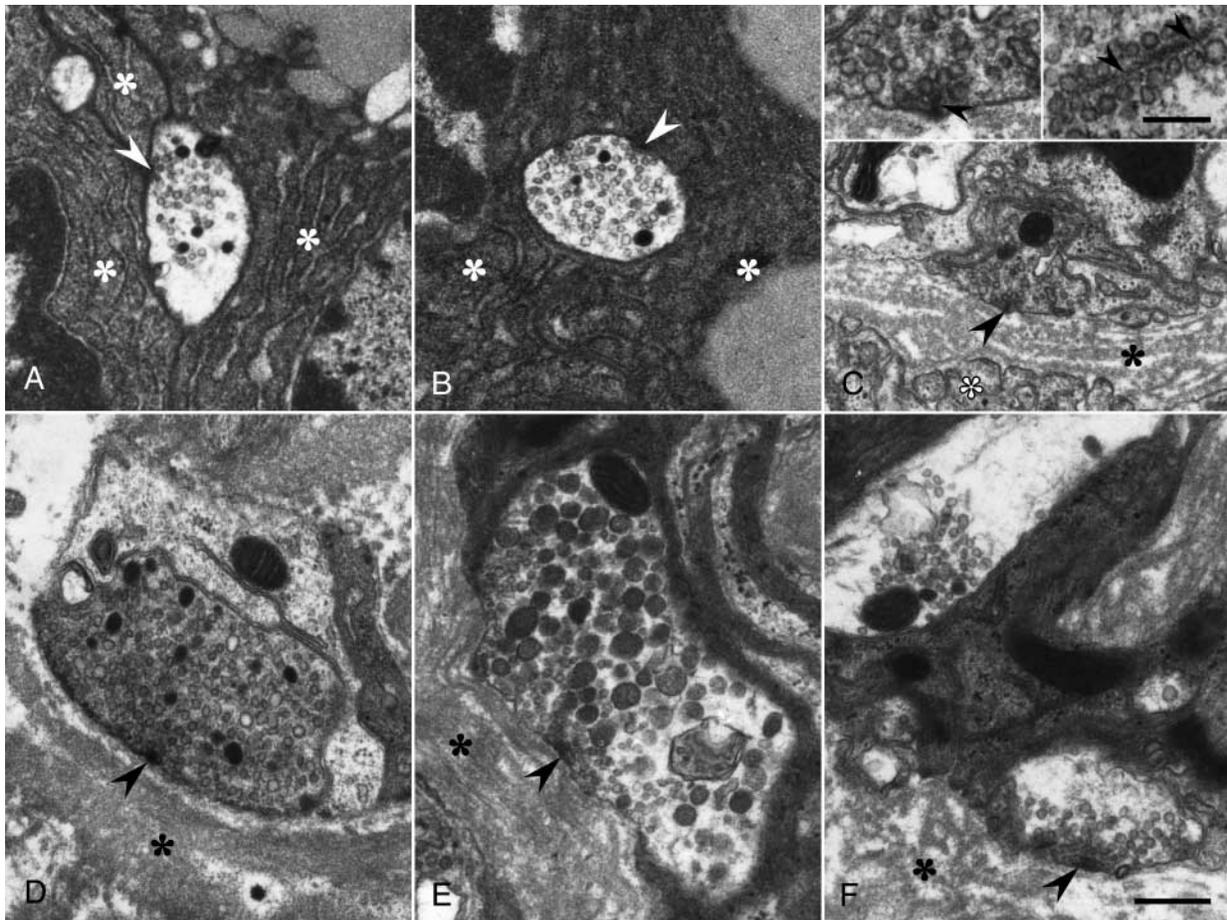

Fig. 7. Ultrastructure of putative release sites for neurotransmitters/neurohormones associated with the salivary gland. (A,B) Axonal profiles embedded between C-cells (asterisks) contain numerous clear vesicles and a few dense vesicles. Small electron-dense areas on the cytoplasmic face of the plasma membrane (white arrowheads) may represent active zones. (C) A putative release site on the outer surface of an acinar lobule, abutting a P-cell (white asterisk). A thick basal lamina (black asterisk) separates the presynaptic membrane from the plasma membrane of the P-cell. (Insets) Ribbon-like electron-dense structures (arrowheads) on the cytoplasmic face of the axonal membrane at higher magnification, in cross-section (left) and *en face* view (right). Rows of vesicles are adjacent to these electron-dense structures, suggesting that they represent the structural correlates of active zones. (D–F) Axonal profiles within nerves that interconnect acinar lobules. On one side, the axonal profiles are without glial wrapping, face a thick basal lamina (asterisks) and have electron-densities on the cytoplasmic face of their plasma membrane (arrowheads). Type A release sites (D,F) have numerous small clear vesicles and few larger dense vesicles. Type B release sites (E) contain fewer clear vesicles and numerous dense vesicles. Scale bars, 0.5 µm (A–F); 0.2 µm (inset).

Baumann et al., 2002). Although numerous varicosities have been observed along the nerve fibres, it has remained uncertain whether all varicosities represent release sites for secretagogues. Electron microscopic analysis, on the other hand, has visualized axonal profiles with structural characteristics of release sites on and within the glands (Whitehead, 1971; Maxwell, 1978); these studies, however, provided no information on the neurotransmitter content or the relative number of release sites associated with the glandular tissue. To circumvent these methodological problems, we double-labelled entire salivary glands with probes for the neurotransmitters serotonin or dopamine, respectively, in order to trace the axons, and with anti-synapsin to identify the release sites. Serial confocal sections through these samples provided insights into the distribution of the release sites for both aminergic secretagogues and their spatial relationship to the various cell types composing the glands. The fine-structural characteristics of the assorted release sites were further determined by electron microscopy, demonstrating the presence of synapses and neurohaemal structures.

The chief findings of these analyses are as follows: (1) varicosities of serotonergic and dopaminergic fibres represent release sites, (2) there are numerous serotonergic synapses within the acini between C-cells, (3) neurohaemal release sites for serotonin were detected next to each pair of P-cells, (4) neurohaemal release sites for dopamine reside occasionally in the immediate vicinity of P-cells, (5) nerves that traverse the acini contain numerous sites for the neurohaemal release of dopamine and few sites for the neurohaemal release of serotonin, (6) few dopaminergic and serotonergic nerve fibres invade the epithelial layer of the salivary ducts and have release sites between the duct cells.

Anti-synapsin as a probe for release sites

The interpretation of our data critically depends on the specificity of anti-synapsin as a marker for release sites. The monoclonal antibody SYNORF1 used in this study was raised against *Drosophila* synapsin (Klagges et al., 1996). Cross-reactivity of this antibody with cockroach synapsin is demonstrated by our western blot analysis. Samples of cockroach brains and salivary glands reveal a single broad band of about 80 kDa that comigrates with the major isoforms of *Drosophila* synapsins. Biochemical assays demonstrate further that the immunoreactive proteins bind to actin filaments, a property shared with synapsins (Valtorta et al., 1992). Finally, the immunoreactive proteins are neuron-specific, as shown by the immunofluorescence labelling of salivary glands.

Since synapsins are universally found on synaptic vesicles at chemical synapses, and since synaptic vesicles are abundant at presynaptic sites, anti-synapsin should provide a good marker for release sites (Valtorta et al., 1992). Control studies with defined synapses and neurohaemal structures, showing an enrichment of anti-synapsin immunoreactivity near the active zones, suggest that ASP foci detected within the salivary gland also represent release sites. The fidelity of the colocalization between anti-synapsin and the varicosities along serotonergic or dopaminergic neurons within the salivary gland endorses the specificity of immunostaining. Furthermore, electron microscopy has revealed numerous chemical synapses and neurohaemal release sites at comparable positions within the salivary gland.

In addition to the punctate staining of varicosities with anti-synapsin, some axons also display homogeneous labelling. This feature is particularly evident on axons with a large diameter, such as those in the salivary nerve (Fig. 3E), and has been noted previously for this antibody in other preparations (Skiebe and Ganeshina, 2000). Axonal labelling may be attributable to synapsin molecules that lie within the cytomatrix or on synaptic vesicle precursors that are *en route* to presynaptic sites (Baitinger and Willard, 1987; Paggi and Petrucci, 1992). Thus, only foci that exhibit intense anti-synapsin immunoreactivity and that are associated with a varicosity are indicative of a release site for neurotransmitter/neurohormone.

Release sites on C-cells are serotonergic

Labelling with anti-synapsin visualizes a high density of release sites in the interior of the acini, between C-cells. Although the outline of C-cells could not be resolved in the phallotoxin images, the high density of ASP structures in the interior of the acini suggests that each C-cell has at least one release site on its surface. In electron microscopic images, equally positioned release sites are frequently observed and are separated only by an extracellular space of a few nanometers in width from the plasma membrane of the C-cells (Maxwell, 1978; present study). Based on morphology, release sites on C-cells can thus be classified as synapses. Colocalization with anti-serotonin but not with anti-TH and the uniform equipment with synaptic vesicles imply further that synapses on C-cells are of the same functional type, i.e. serotonergic. This finding is in accordance with the results of physiological studies, demonstrating that C-cells respond only to serotonin (Just and Walz, 1996). Hence, we conclude that each C-cell is stimulated by serotonin liberated directly at its surface. It remains to be determined whether serotonin receptors on C-cells are clustered in the postsynaptic area or distributed over the plasma membrane.

P-cells have neurohaemal release sites for serotonin and dopamine in their vicinity

P-cells reside at the surface of the acinar lobules and are thus closely juxtaposed to the nerves that entangle the glandular tissue. By labelling with anti-synapsin and by electron microscopy, release sites were identified within these nerves. These sites are separated by a thick basal lamina from the plasma membrane of the P-cells. Secretagogues released from these sites must thus diffuse across an extracellular space of about 0.5 µm in width in order to reach their putative receptors on the P-cell surface. Release sites at this position are heterogeneous with respect to their neurotransmitter content. Serotonergic release sites are detected at each individual pair

of P-cells, whereas dopaminergic release sites are relatively sparse. Electron microscopy confirmed that evenly positioned release sites are diverse regarding their equipment with synaptic vesicles (Maxwell, 1978; present study), but an explicit classification with respect to their transmitter content cannot yet be made.

Nerve fibres around the acinar tissue may not be the only source of secretagogues for the stimulation of P-cells. Since P-cells face the haemolymph with their basal side, they are freely accessible to secretagogues released at some distance by neurohaemal structures, *viz*. the nerves traversing adjacent acinar lobules. Release sites in these interlinking nerves resemble those on the surface of the acinar tissue in their morphology and their equipment with synaptic vesicles (Maxwell, 1978; present study), suggesting a similar transmitter content. In interlinking nerves, however, dopaminergic release sites far outnumber serotonergic release sites.

We thus conclude that P-cells are exposed to serotonin and dopamine liberated at some distance from their surface, in the case of serotonin preferentially at varicosities within nerves on the acinar surface, and in the case of dopamine preferentially at neurohaemal structures between the acinar lobules. The presence of both serotonergic and dopaminergic release sites in the vicinity of P-cells agrees with the view that ion and water transport by this cell type is stimulated by either serotonin or dopamine (Rietdorf et al., 2003).

Serotonergic and dopaminergic release sites are rare and differentially distributed along the salivary duct system

The salivary ducts are sparsely innervated by serotonergic and dopaminergic nerve fibres (Baumann et al., 2002). Surprisingly, these nerve fibres with their release sites are not confined to the outer surface of the ducts, as is the case for other ion-transporting epithelia, but invade deeply into the epithelium. These release sites are, however, infrequent, suggesting that only a minor fraction of the duct epithelial cells is in intimate contact with such sites. Since the duct epithelial cells are extensively coupled by gap junctions (Lang and Walz, 1999b), and since second messenger molecules, such as Ca^{2+} (Lang and Walz, 1999a), can diffuse through gap junctions from an innervated cell to its non-innervated neighbours, direct stimulation of a few epithelial cells may be sufficient to activate ion transport over the entire epithelium. Moreover, since the ducts seem to have free access to the haemolymph, the entire duct system may be exposed to secretagogues liberated at neurohaemal sites, *viz*. the nerves between acinar lobules, and the direct innervation of the duct epithelium may only serve to trigger the response.

Physiological studies on the salivary ducts have shown that dopamine effects ion transport across the epithelial cells, whereas serotonin has no obvious effect (Lang and Walz, 1999a). These observations seem to contradict our finding that the initial segments of the duct system contain both serotonergic and dopaminergic release zones. However, all the physiological studies were performed on areas further

downstream and, thus, on duct segments exclusively innervated by dopaminergic fibres. The presence of serotonergic release sites suggests that duct segments next to the acini differ in their physiological properties from other segments. This hypothesis is in agreement with morphological data demonstrating the presence of secretory granules in duct cells next to acini and the lack of such granules in all other duct cells (Raychaudhuri and Gosh, 1963; Bland and House, 1971). This putative functional diversity of various duct segments warrants further investigation.

Are other neurotransmitters/neurohormones involved with the control of salivation?

Serotonin and dopamine may not be the only secretagogues involved in the control of salivation. In addition to several thin serotonergic axons and a thick dopaminergic axon of salivary neuron 1 (SN1), the salivary nerve contains another thick axon whose cell body is termed SN2 and that resides in the suboesophageal ganglion (Ali, 1997). In locusts, which possess a salivary gland of similar morphology, there is evidence that SN2 contains serotonin (Ali et al., 1993; Ali, 1997) and/or γ-amino butyric acid (GABA; Watkins and Burrows, 1989). In cockroaches, however, SN2 contains neither serotonin nor dopamine (Davis, 1985, 1987; Gifford et al., 1991; Baumann et al., 2002), and whether cockroach SN2 is GABAergic has not yet been examined. The transmitter content and innervation pattern of SN2 is still unknown for the cockroach. If SN2 has varicosities and terminals on or within the glandular tissue, then these should have been labelled with anti-synapsin, and a population of release sites should not have colocalized with anti-serotonin or anti-TH. In our quantitative analysis, release sites that colocalized with anti-dopamine or anti-serotonin on and within the acinar lobules and within interlinking nerves made up roughly 100% of the total number of release sites. We suppose, however, that our quantitative analysis slightly overestimates the number of colocalizing release sites. Provided that an ASP foci is not labelled for serotonin or dopamine but sits next to or in a plane just above a nerve fibre labelled for the secretagogue, the fluorescence signals from both structures may overlap due to the limits of spatial resolution of light microscopy, and such ASP foci may appear in our analysis in the category 'colabelled'. Nevertheless, the above data suggest that release sites of SN2 represent only a minor fraction of the total number of release sites on or within the glandular tissue.

The finding that axonal profiles associated with the salivary gland contain two types of vesicles, *viz*. small clear vesicles and large dense-core vesicles (Whitehead, 1971; Maxwell, 1978; present study) may indicate some additional complexity in the control of salivation. Small clear vesicles are generally considered to contain classical neurotransmitters, whereas large dense-core vesicles may serve the release of neuropeptides (Richmond and Broadie, 2002). It is thus reasonable to assume that neuropeptides are coreleased with serotonin and dopamine in the vicinity of the gland cells. Although the occurrence of various neuropeptides has been

reported in the salivary glands of the locust and the blood-feeding bug, *Rhodnius prolixus* (Myers and Evans, 1985; Tsang and Orchard, 1991; Veelaert et al., 1995; Fusé et al., 1996; Ali, 1997; Te Brugge and Orchard, 2002), our knowledge of the role of neuropeptides in the control of salivation is far from complete. In particular, evidence for the peptidergic innervation of cockroach salivary glands is still lacking and awaits detailed investigation.

References

Ali, D. W. (1997). The aminergic and peptidergic innervation of insect salivary glands. *J. Exp. Biol.* **200**, 1941-1949.

Ali, D. W., Orchard, I. and Lange, A. B. (1993). The aminergic control of locust (*Locusta migratoria*) salivary glands: evidence for serotonergic and dopaminergic innervation. *J. Insect Physiol.* **39**, 623-632.

Baitinger, C. and Willard, M. (1987). Axonal transport of synapsin I-like proteins in rabbit retinal ganglion cells. *J. Neurosci.* **7**, 3723-3735.

Baumann, O. (1997). Biogenesis of plasma membrane domains in fly photoreceptor cells: fine-structural analysis of the cell surface and immunolocalization of Na^+,K^+-ATPase and α-spectrin during cell differentiation. *J. Comp. Neurol.* **382**, 429-442.

Baumann, O. (1998). Association of spectrin with a subcompartment of the endoplasmic reticulum in honeybee photoreceptor cells. *Cell Motil. Cytoskel.* **41**, 74-86.

Baumann, O. (2001). Distribution of nonmuscle myosin-II in honeybee photoreceptors and its possible role in maintaining compound eye architecture. *J. Comp. Neurol.* **435**, 364-378.

Baumann, O. and Lautenschläger, B. (1994). The role of actin filaments in the organization of the endoplasmic reticulum in honeybee photoreceptor cells. *Cell Tissue Res.* **278**, 419-432.

Baumann, O., Dames, P., Kühnel, D. and Walz, B. (2002). Distribution of serotonergic and dopaminergic nerve fibres in the salivary gland complex of the cockroach *Periplaneta americana*. *BMC Physiol.* **2**, 1-15.

Benfenati, F., Valtorta, F., Chieregatti, E. and Greengard, P. (1992). Interaction of free and synaptic vesicle-bound synapsin I with F-actin. *Neuron* **8**, 377-386.

Bland, K. P. and House, C. R. (1971). Function of the salivary glands of the cockroach, *Nauphoeta cinerea*. *J. Insect Physiol.* **17**, 2069-2084.

Davis, N. T. (1985). Serotonin-immunoreactive visceral nerves and neurohemal system in the cockroach *Periplaneta americana* (L.). *Cell Tissue Res.* **240**, 593-600.

Davis, N. T. (1987). Neurosecretory neurons and their projections to the serotonin neurohemal system of the cockroach *Periplaneta americana* (L.), and identification of mandibular and maxillary motor neurons associated with this system. *J. Comp. Neurol.* **259**, 604-621.

Elia, A. J., Ali, D. W. and Orchard, I. (1994). Immunochemical staining of tyrosine hydroxylase (TH)-like material in the salivary glands and ventral nerve cord of the cockroach, *Periplaneta americana* (L.). *J. Insect Physiol.* **40**, 671-683.

Fabian-Fine, R., Höger, U., Seyfarth, E. A. and Meinertzhagen, I. A. (1999a). Peripheral synapses at identified mechanosensory neurons in spiders: three-dimensional reconstruction and GABA immunocytochemistry. *J. Neurosci.* **19**, 298-310.

Fabian-Fine, R., Volknandt, W. and Seyfarth, E. A. (1999b). Peripheral synapses at identifiable mechanosensory neurons in the spider *Cupiennius salei*: synapsin-like immunoreactivity. *Cell Tissue Res.* **295**, 13-19.

Fusé, M., Ali, D. W. and Orchard, I. (1996). The distribution and partial characterization of FMRFamide-related peptides in the salivary glands of the locust, *Locusta migratoria*. *Cell Tissue Res.* **284**, 425-433.

Gifford, A. N., Nicholson, R. A. and Pitman, R. M. (1991). The dopamine and 5-hydroxytryptamine content of locust and cockroach salivary neurones. *J. Exp. Biol.* **161**, 405-414.

Ginsborg, B. L. and House, C. R. (1980). Stimulus-response coupling in gland cells. *Annu. Rev. Biophys. Bioeng.* **9**, 55-80.

Gupta, B. L. and Hall, T. A. (1983). Ionic distribution in dopamine-stimulated NaCl fluid-secreting cockroach salivary glands. *Am. J. Physiol.* **244**, R176-R186.

Just, F. and Walz, B. (1994). Salivary glands of the cockroach, *Periplaneta americana*: new data from light and electron microscopy. *J. Morphol.* **220**, 35-46.

Just, F. and Walz, B. (1996). The effects of serotonin and dopamine on salivary secretion by isolated cockroach salivary glands. *J. Exp. Biol.* **199**, 407-413.

Klagges, B. R. E., Heimbeck, G., Godenschwege, T. A., Hofbauer, A., Pflugfelder, G. O., Reifegerste, R., Reisch, D., Schaupp, M., Buchner, S. and Buchner, E. (1996). Invertebrate synapsins: a single gene codes for several isoforms in *Drosophila*. *J. Neurosci.* **16**, 3154-3165.

Lang, I. and Walz, B. (1999a). Dopamine stimulates salivary duct cells in the cockroach *Periplaneta americana*. *J. Exp. Biol.* **202**, 729-738.

Lang, I. and Walz, B. (1999b). Dye-coupling between cells of the salivary glands in the cockroach *Periplaneta americana*. *Cell Tissue Res.* **298**, 357-360.

Lang, I. and Walz, B. (2001). Dopamine-induced epithelial K^+ and Na^+ movements in the salivary ducts of *Periplaneta americana*. *J. Insect Physiol.* **47**, 465-474.

Maxwell, D. J. (1978). Fine structure of the axons associated with the salivary apparatus of the cockroach, *Nauphoeta cinerea*. *Tissue Cell* **10**, 699-706.

Myers, C. M. and Evans, P. D. (1985). The distribution of bovine pancreatic polypeptide/FMRFamide-like immunoreactivity in the ventral nervous system of the locust. *J. Comp. Neurol.* **234**, 1-16.

Paggi, P. and Petrucci, T. C. (1992). Neuronal compartments and axonal transport of synapsin I. *Mol. Neurobiol.* **6**, 239-251.

Pardee, J. D. and Spudich, J. A. (1982). Purification of muscle actin. *Methods Cell Biol.* **24**, 271-289.

Raychaudhuri, D. N. and Ghosh, S. K. (1963). Study of the histophysiology of the salivary apparatus of *Periplaneta americana* Linn. *Zool. Anz.* **173**, 227-237.

Richmond, J. E. and Broadie, K. S. (2002). The synaptic vesicle cycle: exocytosis and endocytosis in *Drosophila* and *C. elegans*. *Curr. Opin. Neurobiol.* **12**, 499-507.

Rietdorf, K., Lang, I. and Walz, B. (2003). Saliva secretion and ionic composition of saliva in the cockroach *Periplaneta americana* after serotonin and dopamine stimulation, and effects of ouabain and bumetanide. *J. Insect Physiol.* **49**, 205-215.

Skiebe, P. and Ganeshina, O. (2000). Synaptic neuropil in nerves of the crustacean stomatogastric nervous system: an immunocytochemical and electron microscopical study. *J. Comp. Neurol.* **420**, 373-397.

Südhof, T. C. (1995). The synaptic vesicle cycle: a cascade of protein-protein interactions. *Nature* **375**, 645-653.

Te Brugge, V. A. and Orchard, I. (2002). Evidence for CRF-like and kinin-like peptides as neurohormones in the blood-feeding bug, *Rhodnius prolixus*. *Peptides* **23**, 1967-1979.

Tsang, P. W. and Orchard, I. (1991). Distribution of FMRFamide-related peptides in the blood-feeding bug, *Rhodnius prolixus*. *J. Comp. Neurol.* **311**, 17-32.

Valtorta, F., Benfenati, F. and Greengard, P. (1992). Structure and function of the synapsins. *J. Biol. Chem.* **267**, 7195-7198.

Veelaert, D., Schoofs, L., Proost, P., Van Damme, J., Devreese, B., Van Beeumen, J. and De Loof, A. (1995). Isolation and identification of Lom-SG-SASP, a salivation stimulating peptide from the salivary glands of *Locusta migratoria*. *Regul. Pept.* **57**, 221-226.

Watkins, B. L. and Burrows, M. (1989). GABA-like immunoreactivity in the suboesophageal ganglion of the locust *Schistocerca gregaria*. *Cell Tissue Res.* **258**, 53-63.

Whitehead, A. T. (1971). The innervation of the salivary gland in the American cockroach: light and electron microscopic observations. *J. Morphol.* **135**, 483-506.