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Source, topography and excitatory effects of GABAergic innervation in cockroach salivary glands

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SUMMARY

Cockroach salivary glands are innervated by dopaminergic and serotonergic neurons. Both transmitters elicit saliva secretion. We studied the distribution pattern of neurons containing γ-aminobutyric acid (GABA) and their physiological role. Immunofluorescence revealed a GABA-immunoreactive axon that originates within the subesophageal ganglion at the salivary neuron 2 (SN2) and this extends within the salivary duct nerve towards the salivary gland. GABA-positive fibers form a network on most acinar lobules and a dense plexus in the interior of a minor fraction of acinar lobules. Co-staining with anti-synapsin revealed that some putative GABAergic terminals seem to make pre-synaptic contacts with GABA-negative release sites. Many putative GABAergic release sites are at some distance from other synapses and at distance from the acinar tissue. Intracellular recordings from isolated salivary glands have revealed that GABA does not affect the basolateral membrane potential of the acinar cells directly. When applied during salivary duct nerve stimulation, GABA enhances the electrical response of the acinar cells and increases the rates of fluid and protein secretion. The effect on electrical cell responses is mimicked by the $GABA_B$ receptor agonists baclofen and SKF97541, and blocked by the $GABA_B$ receptor antagonists CGP52432 and CGP54626. These findings indicate that GABA has a modulatory role in the control of salivation, acting presynaptically on serotonergic and/or dopaminergic neurotransmission.

Key words: GABA, salivary gland, innervation, insect, cockroach, *Periplaneta americana*.

INTRODUCTION

The control of salivation has been studied intensively in various insect species such as blowflies, cockroaches and locusts. Nevertheless, current knowledge is still fragmentary and does not permit a comprehensive description of the neuronal or hormonal control of salivation (Ali, 1997; Walz et al., 2006). In cockroaches, saliva production by the acinar-type salivary glands is controlled by the nervous system. Two paired neurons within the subesophageal ganglion (SOG), salivary neuron 1 (SN1) and salivary neuron 2 (SN2), send their axons by way of the salivary duct nerve (SDN) towards the salivary gland (Whitehead, 1971; Ali, 1997; Walz et al., 2006; Watanabe and Mizunami, 2006). SN1 contains dopamine as neurotransmitter (Elia et al., 1994) but the neurotransmitter of SN2 is unknown. Besides the SN1 and SN2 axons with a thickness of several micrometers, the SDN contains thinner axons that also project from the SOG towards the salivary gland. These thin axons contain serotonin (5-hydroxytryptamine; 5-HT) as neurotransmitter (Davis, 1985; Baumann et al., 2002; Baumann et al., 2004). An additional source of serotonergic innervation of the salivary gland is provided by the stomatogastric nervous system (Whitehead, 1971; Bowser-Riley et al., 1978; Baumann et al., 2002).

Serotonergic and dopaminergic fibers innervate the secretory tissue of the cockroach salivary glands in different patterns. The salivary glands are composed of three main cell types with different functions (Just and Walz, 1994; Just and Walz, 1996): the grape-like acini consist of a pair of ion-secreting peripheral cells (P-cells) and several protein-secreting central cells (C-cells). The P-cells are innervated by both dopaminergic and serotonergic fibers, and are responsible for ion and water transport. The C-cells are innervated solely by serotonergic fibers and are responsible for protein secretion (Baumann et al., 2002; Baumann et al., 2004). Thus, with dopamine stimulation, a protein-free saliva is produced in the acini whereas 5-HT stimulates the secretion of protein-containing saliva (Just and Walz, 1996; Marg et al., 2004; Rietdorf et al., 2005; Troppmann et al., 2007). The NaCl-rich primary saliva is modified when it passes the extensive duct system. As a result, the final saliva is hypo-osmotic (Gupta and Hall, 1983; Lang and Walz, 2001; Rietdorf et al., 2003; Hille and Walz, 2007).

Although the innervation pattern of cockroach salivary glands has been studied in detail, the neurotransmitter content of SN2 is still unknown. Watkins and Burrows have provided evidence that the SN2 soma in the locust *Schistocerca gregaria* contains GABA (Watkins and Burrows, 1989). Moreover, one thick axon within the SDN of the locust shows GABA immunoreactivity, supporting the view that SN2 is GABAergic (Watkins and Burrows, 1989). The function of GABA in locust salivary glands is still undisclosed. As locust salivary glands are very similar to those of cockroaches with regard to morphology and innervation (Ali, 1997), in the present study, we examined whether there is also GABAergic innervation of the salivary glands in the cockroach *Periplaneta americana*. We determined the source of GABAergic innervation and the spatial relationship of GABA-positive nerve fibers to the various cellular components of the salivary gland complex, and we located putative sites of GABA release on the salivary gland. By intracellular recordings from a nerve–gland preparation and by measuring secretion rates, we obtained information on the possible function of the GABAergic innervation and examined the pharmacological profile of GABA action in this system.

MATERIALS AND METHODS

Animals and preparation

Cockroaches (*Periplaneta Americana* L.) were reared at 25°C under a 12h:12h dark:light cycle and with free access to food and water. Salivary glands of young male imagines were dissected in physiological saline (PS: 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

The following primary antibodies were used: rabbit polyclonal antibody (pAb) anti-GABA (A2052; Sigma, Taufkirchen, Germany), guinea pig pAb anti-GABA (GZ1081; Biotrend, Cologne, Germany), rat monoclonal antibody (mAb) anti-serotonin (MAB352; Chemicon, Hampshire, UK), mouse mAb SYNORF1 against *Drosophila* synapsin (kindly provided by E. Buchner, University of Würzburg, Germany) (Klagges et al., 1996). Secondary antibodies conjugated to Cy3 or Cy5 were obtained from Rockland (Gilbertsville, PA, USA) and Dianova (Hamburg, Germany). Labeling specificity has been demonstrated previously for the antibodies against serotonin and synapsin (Baumann et al., 2002; Baumann et al., 2004). To determine whether labeling for GABA is also specific, cryostat sections of cockroach salivary glands were co-stained with both antisera against GABA; the antisera, although made against different GABA-conjugates (rabbit anti-GABA, GABA conjugated to albumin; guinea pig anti-GABA, GABA conjugated to keyhole limpet hemocyanin) labeled identical structures (data not depicted). Moreover, staining was almost completely abolished by pre-absorption of anti-GABA with 10 mmol l^{-1} GABA but not with 10 mmol l^{-1} glutamate, the substrate of GABA synthesis.

Immunofluorescence labeling

Salivary glands were isolated, fixed for 2 h at room temperature (RT) in 3% paraformaldehyde, 75 mmol l^{-1} lysine-HCl, 10 mmol l^{-1} Na-perjodate, 0.2 mol l^{-1} sucrose, 0.1 mol l^{-1} Na-phosphate buffer (pH 7.0), and washed 3×10 min with phosphate-buffered saline (PBS). Cryosections or entire salivary glands were processed for immunofluorescence labeling as described previously (Baumann et al., 2002; Baumann et al., 2004). Anti-synapsin was applied at a dilution of 1:25, anti-serotonin at a dilution of 1:200, rabbit anti-GABA at a dilution of 1:20,000 or 1:40,000, and guinea pig anti-GABA at a dilution of 1:1000. In order to identify the various cell types and to provide a spatial reference for the position of nerve fibers, specimens were co-labeled with the F-actin probe AlexaFluor488-phalloidin (Invitrogen, Karlsruhe, Germany). Fluorescence images were recorded with a Zeiss LSM 510 confocal microscope (Carl Zeiss, Jena, Germany).

Backfill labeling

The SOG with the SDN was isolated in physiological saline (PS). The nerve was severed and inserted into a droplet of tetramethylrhodamine dextran (TMR-dextran; product number D3308; Invitrogen). After incubation for 48 h at 4°C, specimens were fixed for 2 h at RT as described above, washed 6×20 min with 0.5% Triton-X100 in PBS, dehydrated in a graded ethanol series, cleared with methylsalicylate, rehydrated and the entire ganglia were processed for immunofluorescence labeling with anti-GABA. After dehydration, specimens were embedded in methylsalicylate.

Electrophysiology

The dissected glands were placed in a recording chamber and continuously superfused with PS at a flow rate of ~2 ml min^{-1}. Sharp microelectrodes, used for impaling acinar cells, were filled with 3 mol l^{-1} KCl and had tip resistances of ~70–100 MΩ when inserted into the bath with PS. After impalement the basolateral membrane potential (V_m) of acinar cells was recorded by using a BRAMP 01 bridge amplifier (npi electronic GmbH, Tamm, Germany). The output of the amplifier was displayed on an oscilloscope (Philips PM3331 Combiscope, Kassel, Germany), continuously recorded on a chart recorder (Kipp and Zonen, DELFT BV BD122, Gengenbach Messtechnik, Reichenbach/Fils, Germany) and digitalized at a sampling rate of 1 Hz with a KUSB-3102 A/D-converter (Keithley, Germering, Germany). A/D conversion, data display, storage and output were controlled by TestPoint software (Keithley, Germering, Germany).

Although the microelectrodes were positioned close to an acinus for impalement under optical control through a stereomicroscope, we never knew whether the microelectrode had impaled a P- or a C-cell. We made no attempts in this study to label the cells from which we recorded because P- and C-cells are dye-coupled (Lang and Walz, 1999). For SDN-stimulation, the salivary gland main duct and the attached SDN were taken up into a suction electrode that was coupled to a stimulus isolation unit (SIU 5, Grass Technologies Product Group, Astro-Med GmbH, Rodgau, Germany) and a Grass S48 stimulator. The SDN was stimulated electrically for 2 or 5 s at 5 Hz (resulting in trains of either 10 or 25 stimuli) with each stimulus lasting 0.2 ms in a sufficient strength (5–10 V) to induce acinar cell responses. Prism 4.0 (GraphPad Software, San Diego, CA, USA) was used for statistical analysis. Data were analyzed statistically by using either a paired *t*-test or an analysis of variance (ANOVA) with one-way ANOVA followed by a Tukey post test.

Measurements of fluid and protein secretion

Measurements of fluid secretion were carried out as described previously (Just and Walz, 1996). Briefly, the paired salivary glands were dissected and immediately transferred into the Ringer-filled perfusion bath of a double-chamber having a perfusion bath and a paraffin oil-filled bath, separated by a narrow Vaseline® gap. The perfusion bath was continuously perfused with oxygenated PS. During dissection, the main salivary duct was separated from the adherent reservoir duct and only the salivary duct was drawn through the Vaseline® gap into the oil bath, where droplets of secreted saliva were collected every minute and then transferred into a storage paraffin pool for volume determinations. The volume of the spherical saliva droplets was calculated from their diameter. For electrical stimulation of the salivary nerve, the reservoir duct and the attached SDN were drawn into a suction electrode (in the perfusion chamber), and the glands were stimulated electrically (as described above) sufficiently strong to induce saliva secretion (0.2 ms pulses, 5–10 V, 10 Hz). For statistical analysis, a paired *t*-test was applied to compare rates of fluid secretion in the presence and absence of GABA.

After volume determination, the saliva droplets were transferred to an Eppendorf tube filled with liquid paraffin and stored at –20°C. Protein content of the saliva samples was determined as described previously (Rietdorf et al., 2005) using a modified Bradford assay (Bradford, 1976). For each experiment, saliva droplets from 5 min experimental periods were pooled and diluted with 50 µl H_2O and 200 µl Roti Nanoquant (Roth, Karlsruhe, Germany) working solution. Standards with eight bovine serum albumin concentrations between 0 and 100 µg ml^{-1} were used for calibration. To account

for the small amounts of liquid paraffin, transferred together with the saliva droplets, 5 µl liquid paraffin was added to each standard. The absorption of standards and saliva samples was measured using a GeneQuant™ 1300 spectrophotometer (GE Healthcare, Munich, Germany) to determine protein content. A linear regression was calculated from the data obtained from standard solutions and used for quantification of the protein content in the saliva droplets. A paired *t*-test was used to compare the rates of protein secretion in the absence and presence of GABA.

Chemicals

The GABA receptor ligands (RS)-baclofen, SKF97541 (3-APPA) (both $GABA_BR$ agonists), CGP52432, CGP54626 hydrochloride (both $GABA_BR$ antagonists), muscimol ($GABA_AR$ agonist), picrotoxin, (–)-bicuculline methochloride (both $GABA_AR$ antagonists), (1,2,5,6-tetrahydropyridin-4-yl)-methylphosphinic acid (TPMPA) and 4,5,6,7-tetrahydroisoxazolo[5,4-c] pyridin-3-ol (THIP) (both $GABA_CR$ antagonists, the latter is also a partial $GABA_AR$ agonist) were obtained from Biotrend. GABA, dopamine hydrochloride and 5-HT were obtained from Sigma.

RESULTS

The source of GABAergic innervation

In the locust *Schistocerca gregaria*, SN2 in the SOG provides GABAergic innervation of the salivary gland (Watkins and Burrows, 1989). To examine whether cockroach SN2 is also GABA-positive, the SOG was stained with anti-GABA. To identify SN2, the SDN was cut and backfilled with TMR-dextran. Fig. 1A–D shows the results of backfilling. In the SDN, two axons were stained intensively by TMR-dextran (Fig. 1A, lower right). Upon entering the SOG, the pathways of these axons diverged (Fig. 1A). One axon extended to a soma on the contralateral, anterioventral side of the ganglion, thus representing SN1 (Gifford et al., 1991; Watanabe and Mizunami, 2006). The other TMR-dextran-filled axon could be traced to a soma of ipsilateral, midventral position within the ganglion and represents SN2 (Gifford et al., 1991; Watanabe and Mizunami, 2006). When ganglia were stained with anti-GABA after backfilling, the SN1 soma was labeled only with TMR-dextran whereas the SN2 soma contained TMR-dextran and anti-GABA immunofluorescence (Fig. 1B,C). Other GABA-positive neurons in the SOG were without TMR-dextran signal (Fig. 1D). These results suggest that the SN2 neuron in the SOG of the cockroach contains GABA.

Distribution of GABA-positive fibers over and within the salivary gland complex

Fig. 2A presents a schematic view of the organization of the cockroach salivary gland complex (Just and Walz, 1994) in order to give the reader some landmarks. The paired salivary glands consist of many lobules, each with numerous secretory acini. Every acinus has two fluid-secreting P-cells and several protein-secreting C-cells. The primary saliva produced by the acini passes through an extensive duct system. The salivary ducts of each of the paired glands merge into a single efferent salivary duct. One reservoir is associated with each of the paired glands. The reservoirs open into the reservoir ducts that extend alongside of the efferent salivary ducts and finally fuse to form the main reservoir duct. A reservoir muscle is attached to the orifice of each reservoir.

By labeling isolated salivary glands with anti-GABA, we have analyzed the distribution of GABA-positive nerve fibers in the salivary gland complex and their spatial relationship to the various cell types. In the SDN, one of the two thick axons displayed anti-GABA immunoreactivity (Fig. 2B). As the SDN extended along the reservoir duct, the GABA-positive axon sent off a short thin branch that ran along the outer surface of the reservoir duct (data not shown). The efferent salivary duct and all other salivary ducts, however, were without GABA-positive fibers. Further towards the salivary gland proper, the GABA-positive axon ramified and sent branches to the acinar lobules of the salivary gland, to the reservoir and to the reservoir muscle. In the latter, GABA-immunoreactive fibers formed a network with numerous varicosities (Fig. 2C). This network remained restricted to the portion of the muscle that was affixed to the reservoir. Similarly, GABA-positive fibers were only detected on the basal part of the reservoir, next to the reservoir opening and the attachment site of the muscle (data not depicted). Nerves that interlinked the acinar lobules or that extended from acinar lobules to the reservoir contained GABA-

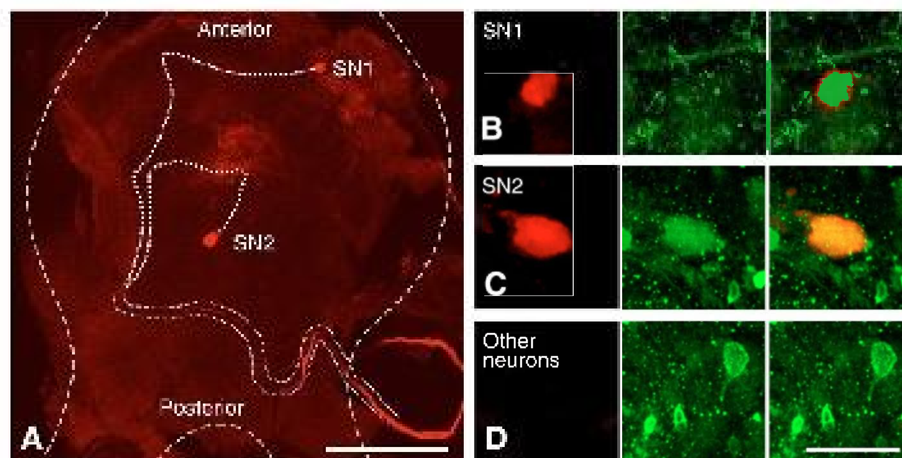

Fig. 1. Anti-GABA labeling of a subesophageal ganglion (SOG, whole-mount) that was backfilled *via* the salivary duct nerve (SDN) with TMR-dextran. (A) A SOG (outline indicated by gray broken lines). The nerve (indicated by gray dotted lines) is partially out of view because it leaves the plane of the optical section and was turned over the ganglion during the embedding procedure. Upon entering the ganglion, the pathways (indicated by white dotted lines) of the two thick axons diverge. One axon extends to a soma with contralateral, anterioventral position (SN1) and the other originates at a soma with ipsilateral, midventral position (SN2). (B–D) Anti-GABA labeling (green) of a SOG that was backfilled with TMR-dextran (red). The right panel shows the composite images. SN1 is labeled only with TMR-dextran (B) whereas SN2 is labeled with both TMR-dextran and anti-GABA (C). Other GABA-positive neurons contain no TMR-dextran (D). Bar A, 250 µm, bar for B,C in D, 50 µm.

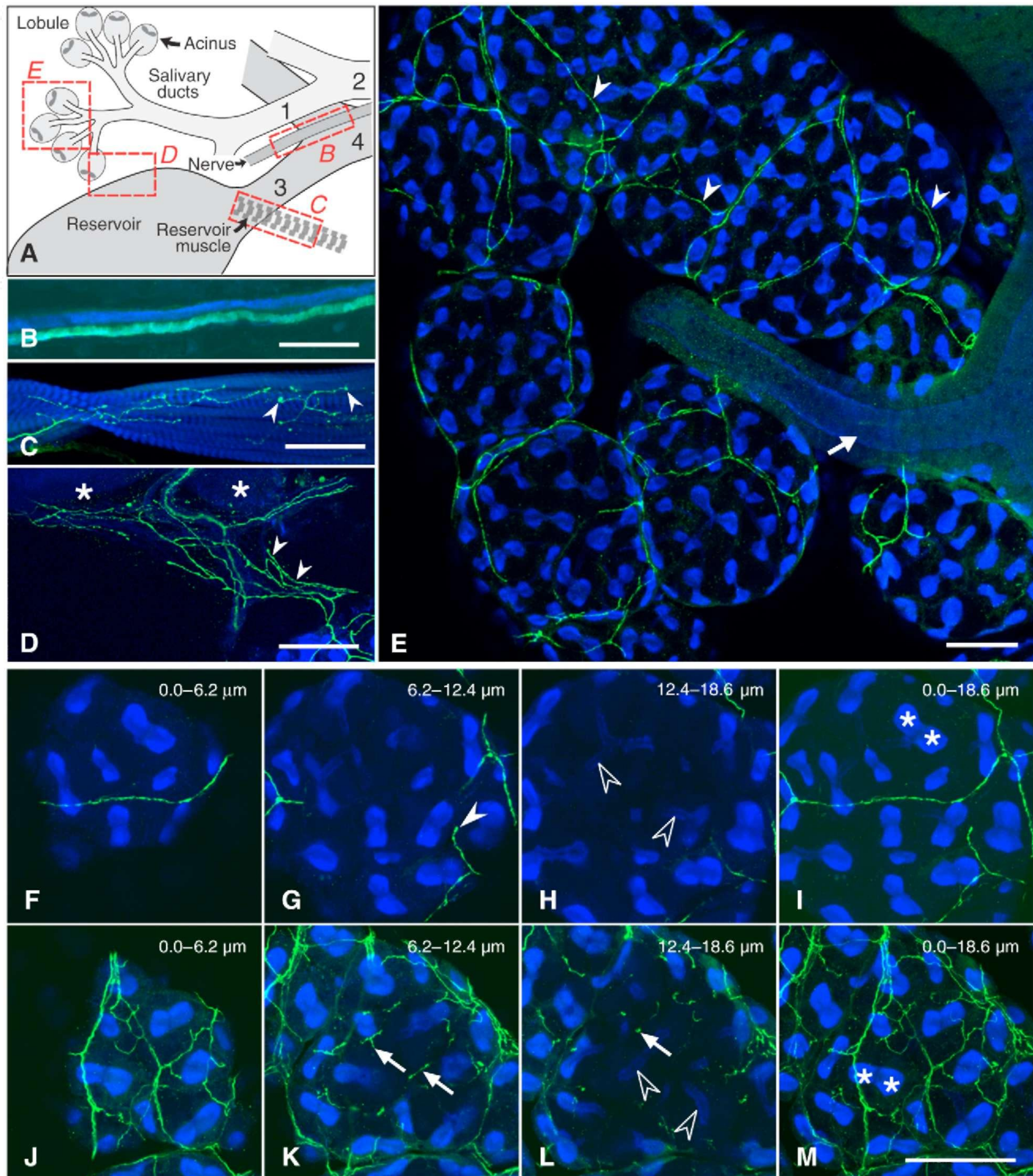

Fig. 2. Distribution of GABA-positive fibers on the various components of the salivary gland complex. Whole-mount preparations of salivary glands were stained with anti-GABA (green) and AlexaFluor488-phalloidin (blue), and imaged by confocal microscopy. (A) A simplified schematic illustration of the organization of the salivary gland complex. The paired salivary glands consist of lobules of acinar tissue. The salivary ducts unite to an efferent salivary duct (1) from each gland, and the two efferent salivary ducts unite to a single main salivary duct (2). The paired reservoirs open into reservoir ducts (3) that unite to the main reservoir duct (4). Red rectangles in A outline the areas shown in B–E. (B) The salivary duct nerve (SDN) contains two thick axons, one of them labeled by anti-GABA. (C) Anti-GABA-immunoreactive fibers (arrowheads) in the reservoir muscle. (D) A nerve that interlinks an acinar lobule (lower right) with the reservoir (asterisks). Anti-GABA-positive fibers (arrowheads) branch and have numerous varicosities within the nerve. (E) An acinar lobule and the associated salivary duct (arrow). P-cells are arranged in pairs with their microvilli intensely stained with phalloidin (blue), providing the appearance of 'bow ties'. A loose network of anti-GABA-reactive fibers is associated with the acinar tissue but not the salivary duct (arrow). (F–H,J–L) Two series of confocal sections through acinar lobules. Each image shows the sum of 17 consecutive optical sections, representing a total thickness of 6.2 µm. The labelling in the upper right indicates the plane of the optical section. (I,M) The sum of all images. P-cells are indicated by asterisks. C-cells in the interior of the acinar lobules are identified by short phalloidin-stained microvilli (open arrowheads). In the lobule shown in (F–I), GABA-positive fibers remain and terminate (arrowhead in G) on the surface of the lobule. In (J–M), GABA-positive fibers extend deep into the acinar lobules (arrows). All bars, 50 µm; bar in M is for F–M.

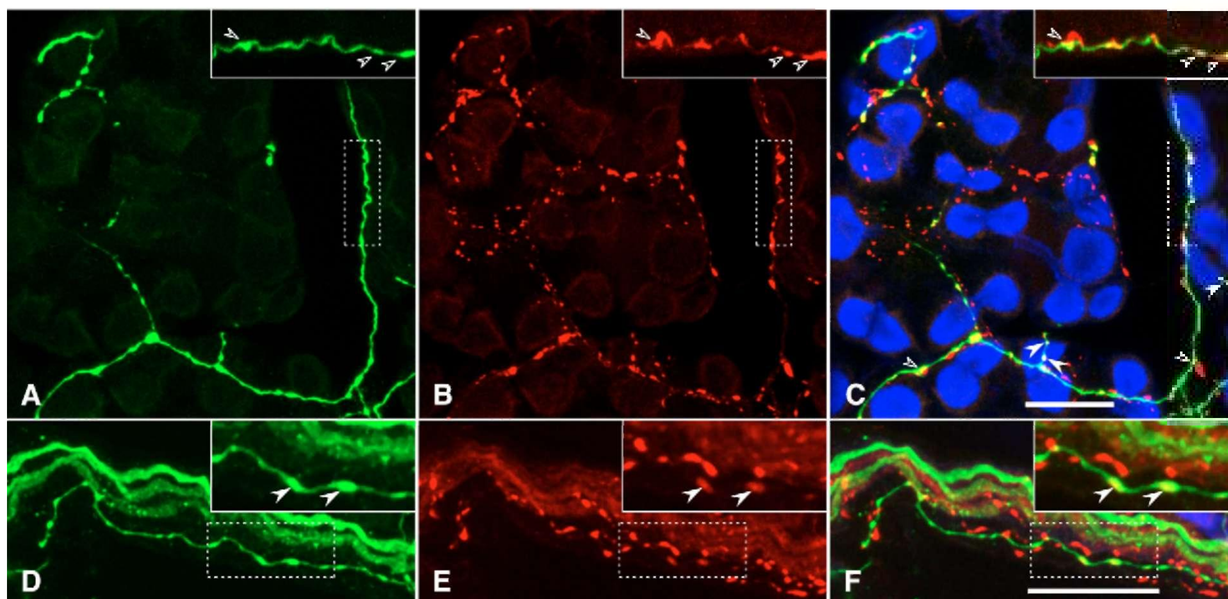

Fig. 3. Distribution of putative release sites for GABA on the salivary gland complex. Whole-mount preparations of salivary glands were stained with anti-GABA (green), anti-synapsin (red) and AlexaFluor488-phalloidin (blue), and imaged by confocal microscopy. Broken-lined rectangles outline the areas that are shown at higher magnification in the insets on the upper right of each image. Putative release sites for GABA are labeled by anti-GABA and anti-synapsin and are indicated by yellow staining in the composite images on the right. Red foci on the composite images may represent release sites for other neurotransmitters. (A–C) Putative release sites for GABA on an acinar lobule. Some putative GABA release sites (open arrowheads) are juxtaposed, others (arrowheads) reside at distance to release sites for other neurotransmitters. (D–F) Putative release sites for GABA in a nerve that interlinks acinar lobules. Most putative release sites for GABA (arrowheads) keep some distance to release sites for other neurotransmitters. Bars A–F, 25 μm.

positive axonal branches of uniform thickness and thin GABA-positive fibers with numerous varicosities (Fig. 2D). Finally, the acinar lobules were covered by a spacious network of varicose GABA-positive fibers (Fig. 2E). Serial optical sectioning demonstrated that these fibers resided on the outer surface of the acinar lobules, close to P-cells (Fig. 2F–I). Due to the spacing of the fiber network, only a small number of P-cells had a GABA-positive fiber in its immediate vicinity. In some acinar lobules within each salivary gland, however, varicose GABA-positive fibers invaded the tissue to form a dense meshwork (Fig. 2J–M). In these lobules, GABA-positive fibers were embedded between P- and C-cells or were surrounded only by C-cells. Acinar lobules with invading GABA-positive fibers were relatively low in number (<10%) and seemed to be distributed at random in the salivary gland.

It could be argued that the disparity in anti-GABA staining between different lobules may have been an artifact as antibodies may not have had equal access to the tissue. However, if acinar tissue was co-stained with anti-GABA and anti-serotonin, all acinar lobules had a dense network of serotonin-positive fibers in their interior, as reported previously (Baumann et al., 2002; Baumann et al., 2004), but only few lobules contained GABA-positive fibers (data not presented). Moreover, a similar difference in anti-GABA labeling pattern between acinar lobules was observed on cryostat sections that provide equal access for antibodies, independent of the location of the acinar lobule within a salivary gland (data not presented). We thus conclude that acinar lobules differ in their innervation pattern with GABA-positive fibers; most lobules have GABA-positive fibers only on their outer surface, and few lobules are penetrated by these fibers.

Position of putative release sites for GABA

By double-labeling with anti-synapsin and anti-GABA, we examined the distribution of putative release sites for GABA in whole-mount preparations of salivary gland complexes (Fig. 3A–F). Foci with anti-synapsin immunoreactivity were detected along thin GABA-positive fibers and usually correlated with the presence of varicosities (Fig. 3A–F, insets). We term such synapsin foci ‘putative release sites for neurotransmitters’. Thicker GABA-positive fibers with uniform diameter, including the GABA-positive axon in the SDN, displayed only weak homogeneous staining for synapsin (Fig. 3D–F). Putative release sites were present along GABA-positive fibers on the outer surface of the efferent reservoir duct, the outer surface of the reservoir, and within the reservoir muscle (data not depicted). Moreover, thin varicose GABA-positive fibers in association with the acinar lobules (Fig. 3A–C) and in nerves that interlinked the acinar lobules contained putative release sites (Fig. 3D–F). It may thus be concluded that GABA release occurs at all structures of the salivary gland complex that are associated with varicose GABA-positive fibers.

In principle, GABA could act on two different target sites in the salivary gland complex. First, it may directly affect some of the various cell types in the salivary gland complex. Second, it could act presynaptically on release sites of other neurons that innervate the salivary gland complex. If the latter were the case, it may be expected that release sites for GABA have a close spatial relationship to release sites for other neurotransmitters. Thus, in whole-mount preparations double-labeled with anti-GABA and anti-synapsin, GABA-positive synapsin foci should be localized next to GABA-negative synapsin foci. Fig. 3A–C demonstrates that this is in fact the case for many but not all of the putative release sites for GABA.

Does GABA affect the acinar cells directly?

In order to obtain information about the possible functions of the GABAergic innervation, we studied the effects of GABA on isolated salivary glands by intracellular recordings from acinar cells.

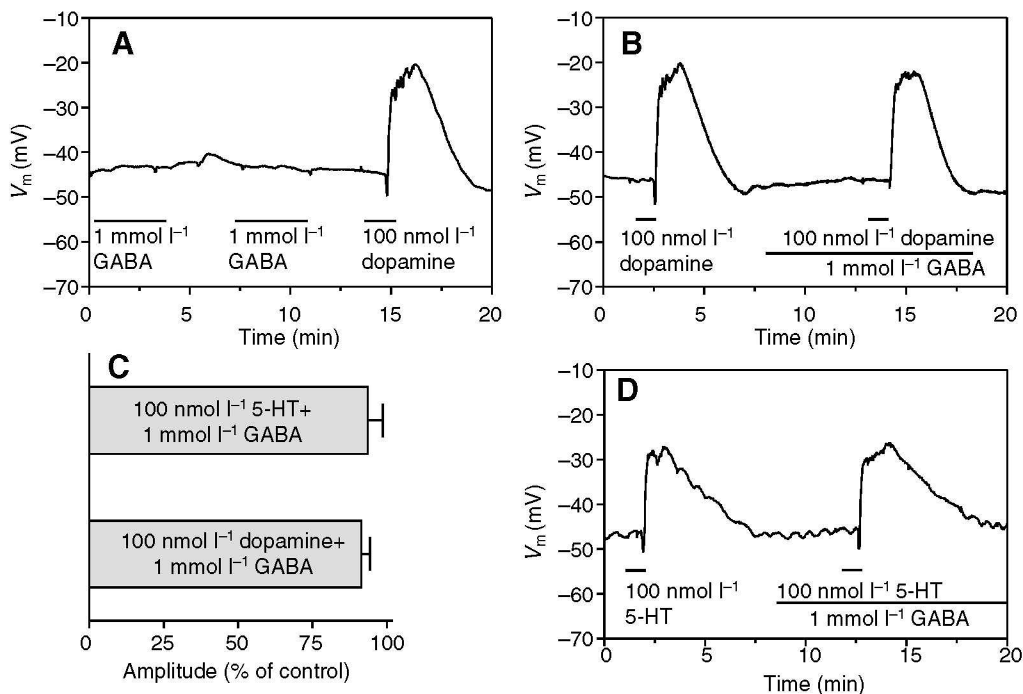

Fig. 4. Changes in the membrane potential (V_m) recorded from acinar cells during bath application of GABA, dopamine and 5-HT. (A) Superfusion of isolated salivary glands with PS containing 1 mmol l^{-1} GABA does not affect the resting V_m of acinar cells. Control application of 100 nmol l^{-1} dopamine evokes multiphasic changes in the V_m of the acinar cell, showing that the isolated gland is functionally intact. (B,D) Co-application of GABA and either dopamine or serotonin (5-HT). 1 mmol l^{-1} GABA has no measurable effect on the amplitudes and kinetics of dopamine- or 5-HT-induced V_m changes in acinar cells. (C) Quantitative summary (means ± s.e.m.) of the experiments illustrated in B and D. Response amplitudes induced by co-application of dopamine or 5-HT and GABA, normalized to control amplitudes with 5-HT or dopamine, respectively, prior to co-application. The amplitude was taken as the range from the hyperpolarization peak to the depolarization peak. 1 mmol l^{-1} GABA did not significantly alter the dopamine- (N=5) and 5-HT-induced (N=6) changes in V_m. Duration of dopamine, 5-HT and GABA applications are indicated by bars.

When isolated salivary glands were superfused with PS containing dopamine or 5-HT, either transmitter induced multiphasic changes in the basolateral V_m of the acinar cells (Fig. 4A,B,D). These electrical responses were composed of a brief initial hyperpolarization (up to 7 mV upon application of 100 nmol l^{-1} dopamine or 5-HT), followed by a large and long-lasting depolarization (up to 25 mV) and sometimes an afterhyperpolarization, irrespectively of whether dopamine or 5-HT was applied (Fig. 4A,B,D). Dose-response curves for dopamine and 5-HT (Fig. 5B) revealed EC_{50} values of 30 nmol l^{-1} and 47 nmol l^{-1}, respectively, when the neurotransmitters were applied in the bathing solution. Bath application of GABA at concentrations up to 1 mmol l^{-1}, however, had no measurable effect on the resting V_m of the acinar cells (Fig. 4A). To determine whether GABA modulates the effects of dopamine and/or 5-HT on the acinar cells, GABA was co-applied with the latter neurotransmitters. Fig. 4B–D illustrates that GABA has no obvious effect on the amplitude or kinetics of the electrical responses induced by sub-saturating concentrations (100 nmol l^{-1}) of dopamine or 5-HT. These results suggest that GABA does not directly affect the basolateral V_m of the acinar cells.

Does GABA act presynaptically?

To examine whether GABA affects the dopaminergic and/or serotonergic neurotransmission, we developed a nerve–gland preparation that permitted electrical stimulation of the SDN *via* a suction electrode and simultaneous intracellular recordings of the basolateral V_m of the acinar cells. Fig. 5A shows the typical changes in the V_m recorded from an acinar cell that was induced by electrical SDN-stimulation. A train of 10 stimuli (0.2 ms, 5 V) applied at a frequency of 5 Hz caused responses that consisted of a hyperpolarization (Fig. 5A, black arrow) of approx. 5±1 mV that was followed by a transient depolarization of approx. 8±1 mV (N=21; means±s.e.m.) (Fig. 5A, open arrow). A comparison of Fig. 5A,C and Fig. 4B,D reveals that the amplitudes and kinetics of stimulus-induced changes in acinar cell V_m were very similar, irrespectively of whether the SDN was electrically stimulated, or whether dopamine or 5-HT were applied in the bath. In fact, the amplitudes of the changes in V_m induced by SDN-stimulation fell into the dynamic range of the response amplitudes generated by bath application of increasing dopamine or 5-HT concentrations (dose-response relationship in Fig. 5B). However, the shape of the electrically induced responses was variable in that the size of the hyperpolarization and the depolarization phase contributed in different amounts to the overall amplitude of the voltage changes (compare Fig. 5A,C, equal size of hyperpolarization and depolarization; Fig. 6C, small hyperpolarization, large depolarization; Fig. 6E, large hyperpolarization, small depolarization). This variability was observed between different preparations, but not during an experiment with one preparation. The factors determining the size of the electrical response components remain unknown but could depend on the cell type that was impaled or on co-release of other neurotransmitters with dopamine and 5-HT.

When the salivary glands were superfused with PS containing 1 μmol l^{-1} GABA, no visible effects on the responses evoked by SDN-stimulation were observed (data not shown). However, upon application of 5 μmol l^{-1} GABA, the amplitudes of the acinar cell responses were almost doubled from 13±1.5 mV to 25±1.8 mV

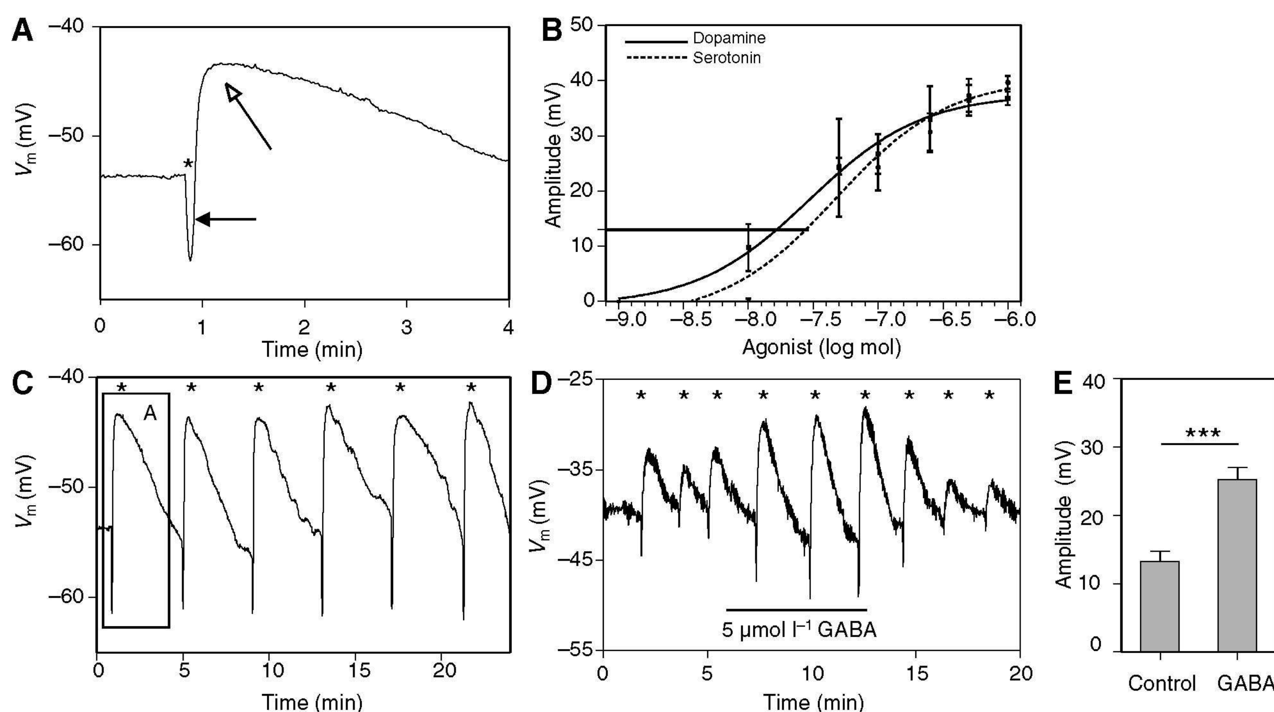

Fig. 5. Membrane potential (V_m) changes in acinar cells induced by electrical salivary duct nerve (SDN)-stimulation (A,C). Asterisks mark the timepoints of electrical stimulation (5 V, 0.2 ms, 5 Hz, 2 s trains). The black arrow in A indicates the hyperpolarization, the open arrow the depolarization induced by one train of electrical stimuli. The box in C indicates the response shown in A. (B) Dose-response relations determined from the changes in V_m induced by bath application of increasing dopamine (black line, means ± s.e.m., N=7) and serotonin (broken line, means ± s.e.m., N=7) concentrations. The mean change in V_m recorded from acinar cells upon electrical SDN-stimulation (13 mV; black line, N=21) is within the dynamic range of the dose-response relations for both neurotransmitters. (D) Bath application of GABA (5 μmol l^{-1}) during SDN-stimulation leads to enhanced amplitudes of the electrical responses recorded from acinar cells. The response amplitudes recover after GABA washout. (E) Comparison of the amplitudes of changes in V_m induced by SDN-stimulation in the absence (control) and in the presence of GABA (N=21, means ± s.e.m.). The mean amplitude of at least two or more consecutive control stimulations was compared by a paired t-test to the mean amplitude of at least two or more stimulations during bath application of GABA. ***P<0.0001.

(N=21, P<0.001) (Fig. 5D,E). This GABA-induced augmentation affected both, the hyperpolarizing and depolarizing phases, and it set in fast, becoming visible in the first electrical stimulation after GABA application. The responses generated by SDN-stimulation in the presence of GABA attained their largest amplitudes after the second or third train of stimuli. After GABA washout, response amplitudes recovered slowly to their initial amplitudes (Fig. 5D). Higher concentrations of GABA (up to 1 mmol l^{-1}) did not further enhance the responses induced by SDN-stimulation. This may be indicative for a narrow dynamic range at GABA concentrations between 1 and 5 μmol l^{-1}.

These data demonstrate that GABA augments the electrical responses of acinar cells only when these are induced by SDN-stimulation, suggesting presynaptic effects on dopaminergic and/or serotonergic neurotransmission.

Pharmacological properties of the GABA effects

To obtain information about the receptor types that are involved in the GABA-mediated effect, we performed pharmacological experiments with agonists and/or antagonists of the various subtypes of GABA receptors. Baclofen (1 mmol l^{-1}) and SKF97541 (5 μmol l^{-1}), agonists of $GABA_B$ receptors ($GABA_BR$) (for reviews, see Bowery et al., 2002; Bettler et al., 2004), mimicked the GABA-induced enhancement of the electrical responses of the acinar cells to SDN-stimulation, with SKF97541 being the more effective agonist (Fig. 6A,B,G). The $GABA_BR$ antagonists CGP54626 (5 μmol l^{-1}) and CGP52432 (5 μmol l^{-1}) suppressed the enhancement of the acinar cell response amplitudes when co-applied with GABA (Fig. 6C,D,G). Likewise, CGP52432 suppressed the SKF97541-induced enhancement of the electrical responses elicited by SDN-stimulation (Fig. 6E,G). These data suggest the involvement of a $GABA_BR$ in the GABA-induced augmentation of the response amplitudes of salivary gland acinar cells that are induced by electrical SDN-stimulation.

With respect to the $GABA_A$ receptor ($GABA_AR$), neither the agonist muscimol (100 μmol l^{-1}) nor the antagonist picrotoxin (100 μmol l^{-1}) affected the V_m changes induced by SDN-stimulation (Fig. 6G). Co-application of picrotoxin and GABA led to enhanced cell responses that did not differ significantly from those induced by GABA alone (Fig. 6G). However, the $GABA_AR$ antagonist bicuculline (5 μmol l^{-1}) enhanced the cell responses significantly when applied alone or when co-applied with GABA, whereby co-application tended to further enhance the response amplitudes, although this effect was not statistically significant (Fig. 6F,G). These data may indicate an additional involvement of an ionotropic GABA receptor.

The $GABA_C$ receptor ($GABA_CR$) specific antagonists TPMPA and THIP (100 μmol l^{-1}) had no significant effect on the response amplitudes when applied alone. When THIP and TPMPA were applied together with GABA, the response amplitudes were not significantly enhanced when compared with the control stimulations but were also not significantly reduced when compared with the GABA effect. (Fig. 6G, first bar) As THIP is also acting as an agonist on $GABA_ARs$ (Waszczak et al., 1980), the tendency to reduce the

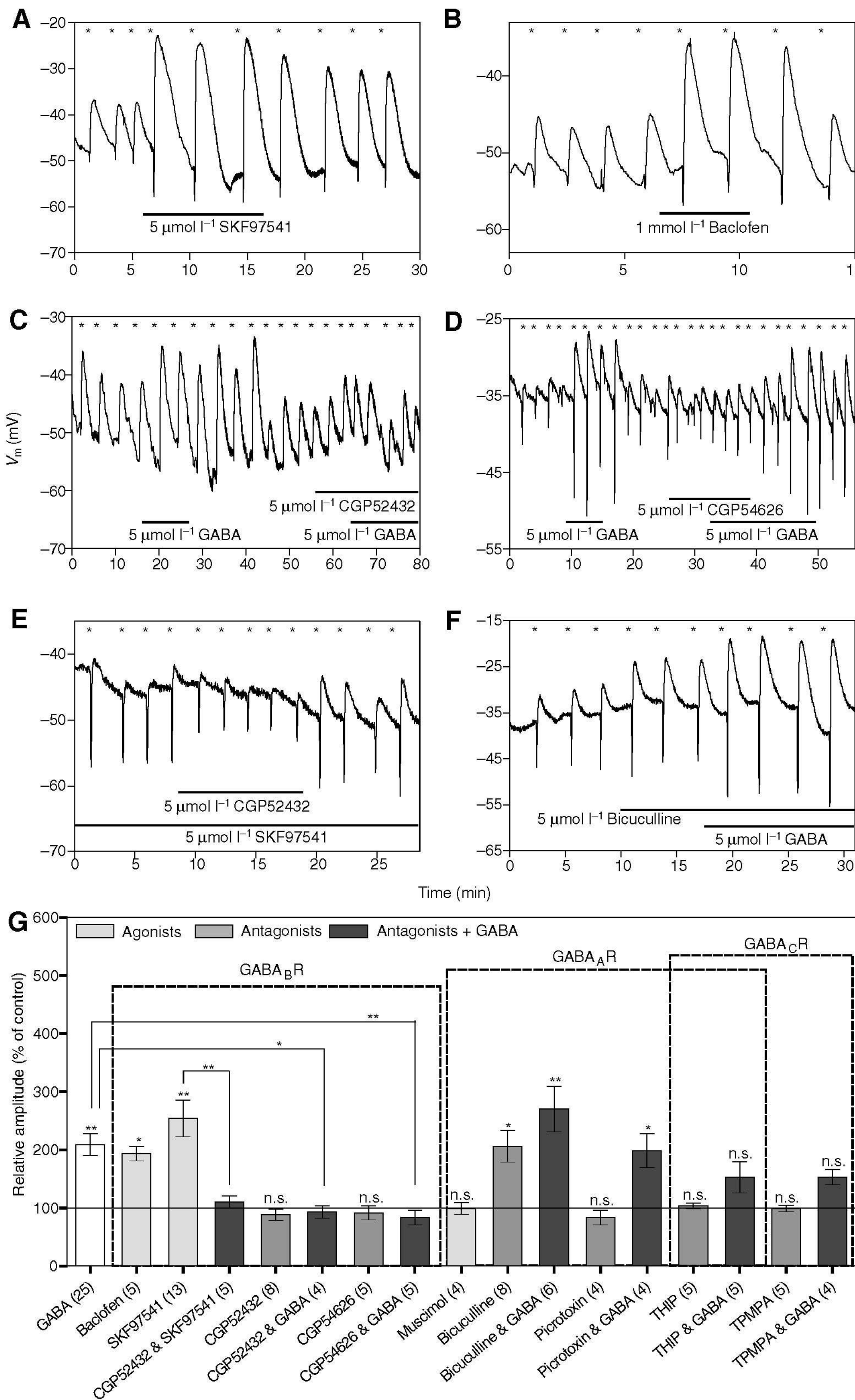

Fig. 6. Effects of agonists and antagonists for various GABA receptor subtypes on the membrane potential (V_m) changes evoked by salivary duct nerve (SDN)-stimulation. Asterisks mark the timepoints of stimulation (5 V, 0.2 ms, 5 Hz, 2 s trains). The duration of drug application is indicated by bars. (A,B) Application of SKF97541 or baclofen mimic the GABA-induced enhancement in the electrical responses. (C,D) CGP52432 and CGP54626 suppress the GABA-induced enhancement. (E) The $GABA_BR$ antagonist CGP52432 suppresses the SKF97541-induced enhancement. (F) Bicuculline-induced augmentation of electrical responses of acinar cells to SDN-stimulation. (G) Bar chart for the effects of all tested drugs. The gray dotted line indicates the control amplitude (=100%) induced by electrical SDN-stimulation in the absence of GABA and other drugs. The number of replicates for each bar is stated in parentheses. The bars show means±s.e.m. Muscimol ($GABA_AR$ agonist), picrotoxin ($GABA_AR$ antagonist), THIP ($GABA_CR$ antagonist and partial $GABA_AR$ agonist) and TPMPA ($GABA_CR$ antagonist) did not affect the control amplitudes or the GABA-induced enhancement. The $GABA_BR$-specific agonists SKF97541 and baclofen enhanced the acinar cell response amplitudes. The $GABA_BR$ antagonists CGP54626 and CGP52432 suppressed the GABA-induced enhancement. Bicuculline also enhanced the cell responses to SDN-stimulation. The mean amplitude of at least two stimulations during GABA-bath application and/or drug treatment was normalized to the mean amplitude of at least two consecutive control stimulations for each experiment. The data were analyzed by applying one-way analysis of variance and Tukey's post test. *P<0.05, **P<0.001.

GABA-induced augmentation of V_m changes induced by SDN-stimulation may be due to an activation of $GABA_AR$s. However, we do not suggest ionotropic receptors of the $GABA_C$ type to be involved in the GABAergic neurotransmission in cockroach salivary glands, as both agonists had no effect when they were applied alone.

Does GABA affect the rates of fluid and protein secretion?

As GABA augmented the electrical changes in membrane potential induced by SDN-stimulation, it was to be expected that it also affects the rate of fluid and protein secretion. In order to test this hypothesis, we measured the rates of fluid and protein secretion during SDN-

stimulation in the absence and presence of GABA in the bathing solution.

Unstimulated glands did not secrete measurable amounts of saliva, and bath application of 5 μmol l^{-1} GABA did not elicit saliva secretion (not shown). Electrical SDN-stimulation (0.2 ms pulses, 8 V, 10 Hz) induced saliva secretion that reached its maximal rate within 3 min (Fig. 7A). The rate of secretion during this first stimulation period served as a control for gland function, and an identical stimulation regime was repeated at the end of each experiment. In order to test whether GABA affects the rate of fluid secretion, we reduced the stimulation frequency (1–2 Hz) close to threshold for fluid secretion because electrical SDN-stimulation does not only stimulate the thick dopaminergic axon and, perhaps, also thin serotonergic axons in the SDN but probably also stimulates the thick GABAergic axon. After 10 min, 5 μmol l^{-1} GABA was applied in the bath for 5 min (Fig. 7A). During GABA application, the rate of fluid secretion induced by SDN-stimulation increased 2.5-fold from 6.8±2.4 to 16.7±2.4 nl min^{-1} (Fig. 7B). After GABA washout, the rate of secretion decreased (Fig. 7B) to 3.7±0.9 nl min^{-1} (N=7). Thus, GABA does not only augment neurotransmission but also the rate of fluid secretion.

Next, we determined whether electrical SDN-stimulation induces protein secretion, and whether GABA affects the rate of protein secretion. We found that electrical SDN-stimulation induced the secretion of protein-containing saliva (Fig. 7C). Thus, our stimulation regime also excited thin serotonergic axons in the SDN. Protein was secreted at a rate of 2.9±0.7 μg min^{-1} (N=7) (Fig. 7C). In the presence of 5 μmol l^{-1} GABA, the rate of protein secretion was enhanced 4-fold to 12±3.8 μg min^{-1} (N=7) (Fig. 7C). Thus, GABA affects both, the rates of fluid and protein secretion.

DISCUSSION

It is well established that the cockroach salivary gland is innervated by dopaminergic and serotonergic neurons (Davis, 1985; Gifford et al., 1991; Elia et al., 1994; Baumann et al., 2002; Baumann et al., 2004), and that the release of dopamine and 5-HT by these neurons elicits salivation (Bowser-Riley and House, 1976; Just and Walz, 1996). However, based on morphological data, it had to be assumed that additional neurotransmitters and/or neurohormones are released at the cockroach salivary gland. First, the SDN contains two thick axons and several thin ones (Whitehead, 1971; Baumann et al., 2004; Watanabe and Mizunami, 2006). One thick axon (SN1) is dopaminergic whereas the thin axons are serotonergic (Davis, 1985; Baumann et al., 2002; Baumann et al., 2004), leaving the neurotransmitter content of the second thick axon (SN2) unidentified. Second, there are at least two different types of nerve terminals on the salivary gland, as judged by the morphology of their synaptic vesicles, and each terminal contains clear vesicles and dense-core vesicles (Maxwell, 1978; Baumann et al., 2004). Thus, co-transmitters may be released along with dopamine and serotonin at the salivary gland.

In accordance with the above hypothesis, the present study provides evidence for the presence of an additional neurotransmitter in nerve fibers that innervate the salivary glands of the cockroach, viz. GABA. The question of whether or not GABA is a co-transmitter of serotonergic and/or dopaminergic neurons could be unequivocally resolved by back-tracing the GABAergic neuron along the SDN to its soma in the SOG. GABA is present only in one thick axon within the SDN and this axon originates from the SN2 soma. Moreover, the distribution of GABA-positive nerve fibers over the salivary gland complex displays differences to both the dopaminergic and the serotonergic innervation pattern (Fig. 7).

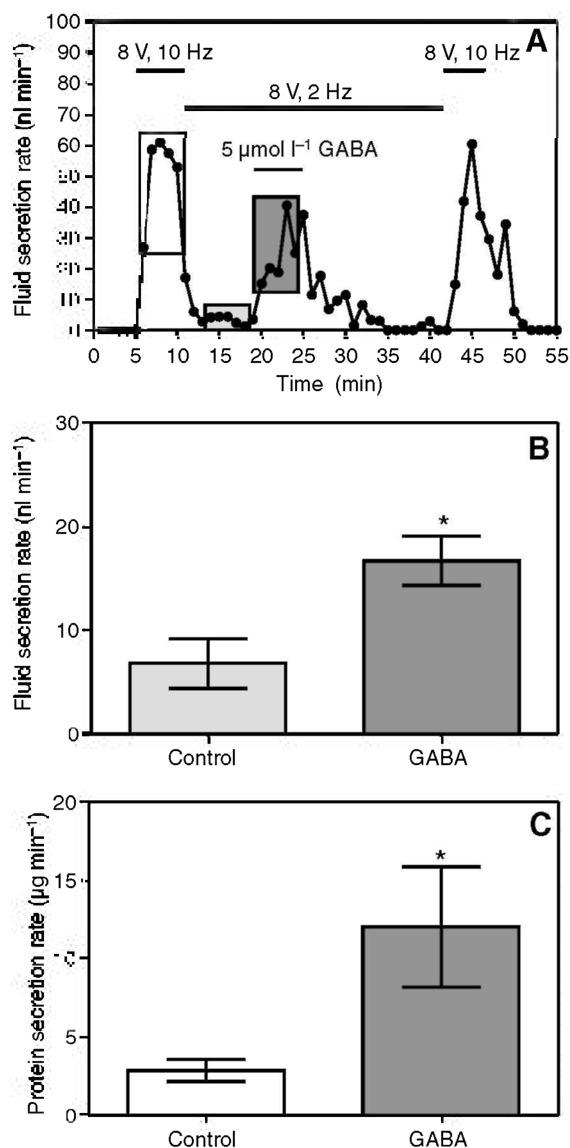

Fig. 7. Effects of GABA on the rates of fluid and protein secretion induced by electrical salivary duct nerve (SDN)-stimulation. (A) Experimental regime and fluid secretion induced by electrical SDN-stimulation in the absence (8 V, 0.2 ms stimuli applied at 10 Hz) and presence (8 V, 0.2 ms stimuli applied at 2 Hz) of GABA. 5 μmol l^{-1} GABA increased the rate of fluid secretion induced by a low frequency of electrical stimuli. After GABA washout the gland was stimulated at a frequency of 10 Hz again to obtain a control for gland functionality. The open rectangle indicates the five saliva samples/time periods that served as control for the protein assay in C. The light gray rectangle labels the five saliva samples/time periods that served as a control for the comparison of the secretion rates in B. The dark gray rectangle indicates the saliva samples/time period when GABA was applied. Application times are indicated by horizontal bars. (B) 5 μmol l^{-1} GABA increases the rate of fluid secretion 2.5-fold from 6.8±1.1 to 16.7±1.7 nl min^{-1} (N=7, *P<0,0171). (C) 5 μmol l^{-1} GABA increases the rate of protein secretion from 2.9±0.7 μg min^{-1} 4-fold to 12±3.8 μg min^{-1} (N=7, *P=0.03).

GABA is thus not a co-transmitter of dopaminergic or serotonergic neurons but GABAergic fibers provide a third source of innervation for the salivary gland.

The GABAergic innervation pattern of the acinar tissue parallels neither the serotonergic nor the dopaminergic innervation pattern.

Serotonergic fibers form a dense network over and within each acinar lobule whereas the salivary duct system, except for short segments next to acinar lobules, lacks serotonergic innervation (Baumann et al., 2002; Baumann et al., 2004). Dopaminergic fibers form a loose network on the outer surface of acinar lobules and ramify within nerves that interlink acinar lobules. Moreover, dopaminergic fibers are present on the salivary duct system over its entire length (Davis, 1985; Baumann et al., 2002; Baumann et al., 2004). GABAergic fibers also form a loose meshwork on the outer surface of most acinar lobules. In contrast to the dopaminergic system, however, GABAergic fibers extend into a few lobules to terminate between C-cells. GABAergic fibers also ramify within nerves that interlink acinar lobules, although not as extensively as the dopaminergic fibers, and the entire salivary duct system lacks GABA-positive fibers.

The finding that acinar lobules differ in their anti-GABA labeling pattern was quite unexpected because serotonergic and dopaminergic innervation does not display such variability (Baumann et al., 2002; Baumann et al., 2004). This raises the question of whether these lobules also differ in other attributes. So far, evidence in favour or against this possibility is lacking.

What are the targets of GABA action on the salivary gland? In theory, there are two possibilities that do not exclude each other: GABA may act (1) directly on P-cells and/or C-cells, or (2) presynaptically on release sites for serotonin and/or dopamine. To distinguish between these possibilities, we have localized putative sites of GABA release. Using anti-synapsin as a marker, we have shown that some of the putative GABA release sites on the secretory tissue reside next to GABA-negative release sites, suggesting that these are presynaptic to release sites for other neurotransmitters. However, we cannot exclude the possibility that the close apposition is coincidental because direct presynaptic contacts have not been detected on electron micrographs (Maxwell, 1978; Baumann et al., 2004). Conversely, numerous putative GABA release sites have no other putative release sites close by. This especially concerns putative GABA release sites in nerves that interlink acinar lobules, providing further evidence that these structures serve as neurohemal organs (Baumann et al., 2004).

Support for a presynaptic GABA action is provided by: (1) the observation that GABA does not induce fluid secretion and (2) the results of our intracellular recordings from acinar cells. GABA had no direct effect on the resting V_m of acinar cells and it did not affect their electrical responses induced by bath application of sub-saturating concentrations of dopamine or 5-HT. These results agree with earlier observations made on isolated salivary glands of the cockroach *Nauphoeta cinerea*, showing that GABA does not affect the resting V_m of acinar cells (Bowser-Riley and House, 1976). In this study, we found that superfusion of *Periplaneta* salivary glands with GABA augments the electrical responses of acinar cells when these are induced by electrical stimulation of the SDN. It may thus be concluded that GABA does not act directly on the acinar cells but rather modulates dopaminergic and/or serotonergic neurotransmission in the salivary gland.

The GABA-induced augmentation of the electrical responses of the acinar cells and of the rates of fluid and protein secretion induced by electrical stimulation of the SDN was rather unexpected, as GABA is best known for its inhibitory function. It has been reported, however, that GABA can also exert excitatory action in some systems (Beg and Jorgensen, 2003; Gulledge and Stuart, 2003; Stein and Nicoll, 2003; Gisselmann et al., 2004). We noted the narrow range of GABA concentrations that affect electrical responses induced by SDN-stimulation. One has to be aware, however, that SDN-stimulation may lead not only to the release of 5-HT and dopamine but also to the release of GABA. Thus, our assays are not suitable to show or measure the entire dynamic range for GABA action.

In *Nauphoeta*, GABA did not augment but slightly reduced the electrical responses induced in secretory cells by electrical stimulation of the SDN (Bowser-Riley and House, 1976). However, this inhibitory effect in *Nauphoeta* required GABA concentrations of >100 μmol. In our *Periplaneta* nerve-gland preparation, 5 μmol GABA was sufficient for the enhancement of the responses induced by SDN-stimulation.

Which receptors mediate the GABA action in the salivary gland? The effects of GABA on the acinar cell response amplitudes were sensitive to a number of $GABA_BR$-specific ligands and recovered slowly. This indicates the involvement of metabotropic GABA receptors ($GABA_BRs$). Both tested $GABA_BR$ agonists enhanced the acinar cell response amplitudes induced by electrical SDN-stimulation and, thus, mimicked the GABA-induced augmentation. Moreover, the $GABA_BR$ antagonists suppressed the GABA-induced augmentation of the electrical responses to SDN-stimulation. As GABA has potentiating effects in our preparation, we suppose an increased transmitter release from dopaminergic and/or serotonergic release sites. $GABA_BRs$ are described to couple to the cAMP pathway by increasing or decreasing adenylyl cyclase acitivity (Hill, 1985; Olianas and Onali, 1999; Mezler et al., 2001). Elevated presynaptic cAMP can increase transmitter release (Yoshihara et al., 2000; Kaneko and Takahashi, 2004; Cheung et al., 2006). GABA could increase transmitter release from dopaminergic and/or serotonergic release sites on *Periplaneta* salivary glands in a similar way, resulting in enhanced responses of the acinar cells upon SDN-stimulation.

The bicuculline sensitivity of the GABA effect indicates that an ionotropic GABA receptor ($GABA_AR$) may be involved in addition to the $GABA_BR$. $GABA_ARs$ are known to inhibit neurotransmission by increasing chloride currents that hyperpolarize the pre- or postsynaptic membrane and thus reduce transmitter release and/or excitability (for reviews, see MacDermott et al., 1999; Engelmann and MacDermott, 2004). The enhancement of acinar cell responses by the $GABA_AR$ antagonist bicuculline supports this view.

The salivary glands in *Periplaneta* are innervated by serotonergic and dopaminergic neurons (Davis, 1985; Elia et al., 1994; Baumann et al., 2002; Baumann et al., 2004), and stimulation by dopamine or serotonin results in the secretion of saliva without or with proteins (Just and Walz, 1996; Marg et al., 2004; Rietdorf et al., 2005; Troppmann et al., 2007). Thus, it must be envisaged that the involved GABA receptor subtypes are located on different neurons so that the release of dopamine and serotonin is modulated by GABA *via* an ionotropic or a metabotropic GABA receptor differentially. Because GABA enhanced the rate of protein secretion 4-fold, it seems likely that $GABA_BR$ are located at least on the serotonergic neurons. The unequivocal identification of the target neuron(s) for GABA action will require the localization of the GABA receptors.

Besides the acinar tissue, GABAergic fibers and putative GABA release sites are also associated with the reservoir system including the reservoir muscle. The functions of the reservoir system are still enigmatic. It may be a storage compartment for saliva but it could also have osmoregulatory function (Raychaudhuri and Gosh, 1963; Sutherland and Chillseyzn, 1968). The reservoir muscle may act as an occlusor of the reservoir orifice and, as the muscle relaxes, hemolymph pressure may cause compression of the reservoir walls and emptying of the reservoir (Sutherland and Chillseyzn, 1968).

In view of this scenario and in view of the confinement of GABAergic fibers to the orifice region of the reservoir and of the reservoir muscle, this fiber system could also be involved in the regulation of reservoir emptying.

In view of these findings, it may be concluded that GABA acts on cockroach salivary glands as a neuromodulator rather than as a classical neurotransmitter. This GABA action is very likely to be functionally important for the regulation of salivation and saliva composition because SN2 displays spontaneous spike activity that increases during feeding (Watanabe and Mizunami, 2006).

LIST OF ABBREVIATIONS

C-cell	central cell
5-HT	serotonin
P-cell	peripheral cell
PBS	phosphate buffered saline
PS	physiological saline
SDN	salivary duct nerve
SN1	salivary neuron 1
SN2	salivary neuron 2
SOG	subesophageal ganglion
V_m	membrane potential

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REFERENCES

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

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

Baumann, O., Kühnel, D., Dames, P. and Walz, B. (2004). Dopaminergic and serotonergic innervation of cockroach salivary glands: distribution and morphology of synapses and release sites. *J. Exp. Biol.* **207**, 2565-2575.

Beg, A. A. and Jorgensen, E. M. (2003). EXP-1 is an excitatory GABA-gated cation channel. *Nat. Neurosci.* **6**, 1145-1152.

Bettler, B., Kaupmann, K., Mosbacher, J. and Gassmann, M. (2004). Molecular structure and physiological functions of $GABA_B$. receptors. *Physiol. Rev.* **84**, 835-867.

Bowery, N. G., Bettler, B., Froestl, W., Gallagher, J. P., Marshall, F., Raiteri, M., Bonner, T. I. and Enna, S. J. (2002). International Union of Pharmacology. XXXIII. Mammalian γ-aminobutyric $acid_B$ receptors: structure and function. *Pharmacol. Rev.* **54**, 247-264.

Bowser-Riley, F. and House, C. R. (1976). The actions of some putative neurotransmitters on the cockroach salivary gland. *J. Exp. Biol.* **64**, 665-676.

Bowser-Riley, F., House, C. R. and Smith, R. K. (1978). Competitive antagonism by phentolamine of responses to biogenic amines and the transmitter at a neuroglandular junction. *J. Physiol. (Lond.)* **279**, 473-489.

Bradford, M. M. (1976). A rapid and sensitive methof for the quantification of microgramm quantities of protein utilizing the principle of protein-dye-binding. *Anal. Biochem.* **72**, 15-23.

Cheung, U., Atwood, H. L. and Zucker, R. S. (2006). Presynaptic effectors contributing to cAMP-induced synaptic potentiation in *Drosophila*. *J. Neurobiol.* **66**, 273-280.

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

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.

Engelman, H. S. and MacDermott, A. B. (2004). Presynaptic ionotropic receptors and control of transmitter release. *Nat. Rev. Neurosci.* **5**, 135-145.

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.

Gisselmann, G., Plonka, J., Pusch, H. and Hatt, H. (2004). *Drosophila melanogaster* GRD and LCCH3 subunits form heteromultimeric GABA-gated cation channels. *Br. J. Pharmacol.* **142**, 409-413.

Gulledge, A. T. and Stuart, G. J. (2003). Excitatory actions of GABA in the cortex. *Neuron* **37**, 299-309.

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.

Hill, D. R. (1985). $GABA_B$ receptor modulation of adenylate cyclase activity in rat brain slices. *Br. J. Pharmacol.* **84**, 249-257.

Hille, C. and Walz, B. (2007). A vacuolar-type H^+-ATPase and a Na^+/H^+ exchanger contribute to intracellular pH regulation in cockroach salivary ducts. *J. Exp. Biol.* **210**, 1463-1471.

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.

Kaneko, M. and Takahashi, T. (2004). Presynaptic mechanism underlying cAMP-dependent synaptic potentiation. *J. Neurosci.* **24**, 5202-5208.

Klagges, B. R., 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. (1999). Dopamine stimulates salivary duct cells in the cockroach, *Periplaneta americana*. *J. Exp. Biol.* **202**, 729-738.

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.

MacDermott, A. B., Role, L. W. and Siegelbaum, S. A. (1999). Presynaptic ionotropic receptors and the control of transmitter release. *Annu. Rev. Neurosci.* **22**, 443-485.

Marg, S., Walz, B. and Blenau, W. (2004). The effects of dopamine receptor agonists and antagonists on the secretory rate of cockroach (*Periplaneta americana*) salivary glands. *J. Insect Physiol.* **50**, 821-830.

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

Mezler, M., Müller, T. and Raming, K. (2001). Cloning and functional expression of $GABA_B$ receptors from *Drosophila*. *Eur. J. Neurosci.* **13**, 477-486.

Olianas, M. C. and Onali, P. (1999). $GABA_B$ receptor-mediated stimulation of adenylyl cyclase activity in membranes of rat olfactory bulb. *Br. J. Pharmacol.* **126**, 657-664.

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.

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.

Rietdorf, K., Blenau, W. and Walz, B. (2005). Protein secretion in cockroach salivary glands requires both, an increase in Ca^{2+} and cAMP concentrations. *J. Insect Physiol.* **51**, 1083-1091.

Stein, V. and Nicoll, R. A. (2003). GABA generates excitement. *Neuron* **37**, 375-378.

Sutherland, D. J. and Chillseyzn, J. M. (1968). Function and operation of cockroach salivary reservoir. *J. Insect Physiol.* **14**, 21-31.

Troppmann, B., Walz, B. and Blenau, W. (2007). Pharmacology of serotonin-induced salivary secretion in *Periplaneta americana*. *J. Insect Physiol.* **53**, 774-781.

Walz, B., Baumann, O., Krach, C., Baumann, A. and Blenau, W. (2006). The aminergic control of cockroach salivary glands. *Arch. Insect Biochem. Physiol.* **62**, 141-152.

Waszczak, B. L., Hruska, R. E. and Walters, J. R. (1980). GABAergic actions of THIP *in vivo* and *vitro*: a comparison with muscimol and GABA. *Eur. J. Pharmacol.* **65**, 21-29.

Watanabe, H. and Mizunami, M. (2006). Classical conditioning of activities of salivary neurones in the cockroach. *J. Exp. Biol.* **209**, 766-779.

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.

Yoshihara, M., Suzuki, K. and Kidokoro, Y. (2000). Two independent pathways mediated by cAMP and protein kinase A enhance spontaneous transmitter release at *Drosophila* neuromuscular junctions. *J. Neurosci.* **20**, 8315-8322.