Synaptic Structure, Distribution, and Circuitry in the Central Nervous System of the Locust and Related Insects

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ABSTRACT The Orthopteran central nervous system has proved a fertile substrate for combined morphological and physiological studies of identified neurons. Electron microscopy reveals two major types of synaptic contacts between nerve fibres: chemical synapses (which predominate) and electrotonic (gap) junctions. The chemical synapses are characterized by a structural asymmetry between the pre- and postsynaptic electron dense paramembranous structures. The postsynaptic paramembranous density defines the extent of a synaptic contact that varies according to synaptic type and location in single identified neurons. Synaptic bars are the most prominent presynaptic element at both monadic and dyadic (divergent) synapses. These are associated with small electron lucent synaptic vesicles in neurons that are cholinergic or glutamatergic (round vesicles) or GABAergic (pleomorphic vesicles). Dense core vesicles of different sizes are indicative of the presence of peptide or amine transmitters. Synapses are mostly found on small-diameter neuropilar branches and the number of synaptic contacts constituting a single physiological synapse ranges from a few tens to several thousand depending on the neurones involved. Some principles of synaptic circuitry can be deduced from the analysis of highly ordered brain neuropiles. With the light microscope, synaptic location can be inferred from the distribution of the presynaptic protein synapsin I. In the ventral nerve cord, identified neurons that are components of circuits subserving known behaviours, have been studied using electrophysiology in combination with light and electron microscopy and immunocytochemistry of neuroactive compounds. This has allowed the synaptic distribution of the major classes of neurone in the ventral nerve cord to be analysed within a functional context. Microsc. Res. Tech. 56:210–226, 2002. © 2002 Wiley-Liss, Inc.

INTRODUCTION

The brain and ventral nerve cord of locusts and other insect groups have provided important model systems for the analysis of the neural networks underlying both simple and complex behaviour. One of the key factors that has contributed to the interpretability of insect neuronal circuitry is that it incorporates many neurones that are uniquely identifiable in terms of their morphology and their physiological characteristics. This allows a synergistic approach to the analysis of circuitry, in which the physiological properties and synaptic distribution of single neurones can be viewed in the context of the behaviour that the circuit subserves. Among insects, the locust and other large Orthopterans are particularly well suited to this approach because of their large size and the accessibility of the nervous system. The Orthopteran nervous system also shares many properties with that of Diptera such as Drosophila, in which such studies are still largely impracticable but whose suitability for genetic manipulation has led to insights into the molecular biology of insect neurones and their synaptic equipment (Prokop, 1999).

This review focuses on the morphology and distribution of synapses viewed in the context of synaptic circuitry and is based primarily on information derived from the locust nervous system. However, it also draws insights from studies of other insects. We provide a brief review of synaptic ultrastructure of both chemical and electrical synapses, and also some information on putative non-synaptic release of neurotransmitters from as yet unpublished data. The distribution and abundance of synaptic contacts on different classes of neurones are considered in some detail and we present examples of the complex synaptic microcircuitry in which certain individual physiologically characterised neurones are involved. The structural dynamics of synapses during pre- and postembryonic development, and during degeneration and regeneration, are outside the scope of this review. For an analysis of these topics, the reader is referred to the work of Budnik (1996), Davis and Goodman (1998), Meinertzhagen (1993), Meinertzhagen and Hu (1996), and Prokop (1999).

SYNAPTIC ULTRASTRUCTURE

Chemical Synapses

The contacts between neurones that are interpreted as being the sites of chemical synapses typically share a number of structural features: synaptic vesicles as-
associated with presynaptic electron dense membrane appositions, a widened synaptic cleft, and a sub-membranous electron-dense coating in the opposing postsynaptic elements (Figs. 1–3; see also Figs. 5,6). These basic components have been frequently observed in locusts and other insect forms using conventional electron microscopy (Boeckh et al., 1970; Burkhardt and Braithen, 1976; Osborne, 1966; Schürmann, 1980; Schürmann and Wechsler, 1969, 1970; Tyer and Altman, 1976; Wood et al., 1977). The sub-membranous densities and the material within the synaptic cleft are best visualised in aldehyde fixed, non-osmicated tissue (Schürmann, 1980) in which the membranes themselves are left unstained (Fig. 1C). A single presynaptic terminal may lie opposite a single postsynaptic neurite (a monadic synapse) or more commonly, a pair of postsynaptic processes (a dyadic synapse) (e.g., Watson and Burrows, 1982).

In insects, neuronal somata generally lie outside the neuropile and are heavily ensheathed in glial cells. However, synapse-like specialisations made by dopaminergic neurones are common on Kenyon cell somata in the mushroom bodies of bees (Blenau et al., 1999). Comparable studies on Orthopteran Kenyon cells have yet to be carried out. While the postsynaptic element at central chemical synapses is almost universally another neurone, occasionally this can be demonstrated as a glial cell process (Fig. 1F) (Watson and Burrows, 1989). The functional significance of these different presynaptic structures, typically ranges from about half a micrometer to a few micrometers in length and, depending on fixation, a narrow plate may be seen running above it (Foelix and Choms, 1979; Watson and Burrows, 1982; Wood et al., 1977). This differs significantly from the presynaptic bars seen at synapses in the fly brain, at which the bar has a prominent broad plate giving it a "T" shaped profile in cross-section (Burkhardt and Braithen, 1976; Fröhlich and Meinertzhagen, 1983). A presynaptic grid of regularly arranged plated columns is found at many synapses in the bee brain (Schürmann, 1971). The functional significance of these different presynaptic figures remains obscure. At some dyadic synapses, two or even all three of the neurites involved may contain a presynaptic bar (Burrows et al., 1989; Watson and Pfüger, 1984). Though numerically uncommon, such forms are locally abundant in certain neuropiles (e.g., of the mushroom bodies) or at synapses involving certain identified neurones (Burrows et al., 1989; see below). As can be seen from contacts between the DCMD/LGMD neurones in the locust brain (Killmann et al., 1999), a single neurone may form both dyadic and monadic synapses with or without presynaptic structures.

Freeze fracture studies of insect central nervous tissue have revealed clusters or rows of what appear to be vesicle attachment sites on either side of troughs that are interpreted as the presynaptic membrane of dyadic synapses (Fröhlich, 1985; Tolbert and Hildebrand, 1981; Wood et al., 1977). Coated pits that may represent the sites of membrane retrieval have also been reported close to the active zone (Lane et al., 1983). The presynaptic membrane in the vicinity of the region of synaptic contact may show an enhanced electron density compared to adjacent regions. However, this is less pronounced than that associated with the postsynaptic membrane (Killmann et al., 1999). At the postsynaptic membrane, the active zone is marked by a submembranous electron dense layer of variable extent (Figs. 1,2). A number of active zones in close proximity may occur along extended regions of membrane apposition between two neurons (Fig. 1F) (Killmann et al., 1999), reminiscent of the distribution of synaptic loci at fly neuromuscular junctions (Feeny et al., 1998). The synaptic cleft has a width of 10–20 nm, which varies depending on synaptic type (Fig. 2) (Killmann et al., 1999). The enhanced electron density of the material in the synaptic cleft and of the submembranous structures of the pre-and postsynaptic membranes is most probably a manifestation of the synaptic proteins associated with chemical transmission and synaptic stabilisation (Garner et al., 2000; Kneussel and Betz, 2000; Prokop, 1999). In the insect nervous system, the identities and functional roles of these synaptic proteins are now under intensive investigation, particularly at the glutamatergic neuromuscular synapses of Drosophila (Prokop 1999). Identification of the precise molecular structures of synaptic proteins at glutamatergic and non-glutamatergic synapses within the locust nervous system requires further molecular and pharmacological investigation.

The synaptic vesicles that lie in closest proximity to the presynaptic densities in the locust central nervous system are generally small and electron lucent and fall into two categories: round with a typical diameter range of 35–50 nm, or pleomorphic with dimensions of 20–30 nm. Pleomorphic vesicles are found almost exclusively in terminals that show evidence for the presence of GABA, while round agranular vesicles are found in terminals that are immunoreactive for glutamate (Watson, 1988; Fig. 3A, B). Neurones that appear to be cholinergic (Leitinger and Simmons, 2000; Lutz and Tyer, 1988) also contain round agranular vesicles (Watson and England, 1991; Simmons and Littlewood, 1989; Watson et al., 1991, 1993). Synaptic terminals containing round agranular vesicles are generally more numerous than those containing pleomorphic vesicles and in ultrastructural immunocytochemical studies of identified interneurones or efferent neurones, about 20–40% of presynaptic terminals that contain electron lucent vesicles exhibit GABA-like immunoreactivity. Following chemical fixation, electron lucent synaptic vesicles are only rarely seen to be undergoing exocytosis as shown for stimulated locust motoneurone-muscle synapses (Reinecke and Walther, 1981). Occasionally evidence of vesicle recycling in the form of clathrin-coated vesicles has been reported in insects (Killmann et al., 1999). Large dense core vesicles with a diameter of 60–120 nm occur in all synaptic neuropiles of Orthopteran insects (Fig. 3C–G). These may be scattered among electron lucent vesicles but more often lie in distinct clusters in the vicinity of the presynaptic membrane. Dense core vesicles are particularly abundant in certain neuropile compartments within the brain (e.g., lateral protocerebrum, central body complex, mushroom bodies, and deutocerebral neuropiles) that may be richly endowed with neuropeptides and biogenic amines (Nässel, 1996; see Homberg, pages
Fig. 1.
taining these classes of transmitters. Peptides can often be demonstrated to be co-localised with the fast-acting transmitters associated with electron lucent vesicles. For example, the peptide proctolin is present in some Orthopteran glutamatergic motorneurones (Adams and O’Shea, 1983; Bartos et al 1994; Keshishian and O’Shea, 1985). In the Orthoptera, biogenic amines such as 5-HT (Elekes and Hustert, 1988; Peters et al., 1987; Peters and Tyrer, 1987), dopamine (Elekes et al., 1987), octopamine (Schürmann et al., 1995), and histamine (Hörner et al., 1996) are found in terminals that make conventional synapses and that contain both dense core vesicles and electron lucent vesicles (Fig. 3C–E). Similar observations have been made in other insect groups (Nässel, 1996). The pre-embedding immunocytochemical methods that have been most commonly used to investigate the ultrastructural distribution of these neurotransmitters do not allow a clear identification of the vesicle populations with which they are associated. The post-embedding immunogold technique has revealed that serotonin is associated with large dense core vesicles in neurosecretory terminals of *Rhodnius* (Miksys and Orchard, 1994). Immunocytochemical evidence for the presence of neuropeptides such as adipokinetic hormones, FMRFamid, allostatin, and proctolin within dense core vesicles has been obtained from studies in the locust (Diederen et al., 1987, pages 227–236, in this volume) and the cockroach (Ude and Agricola, 1995; Ude and Eckert, 1988). In some classes of axon terminals within the nervous system of the locusts and other Orthopterans (Fig. 1D,E), dense core vesicles appear closely associated with synapse-like active zones (Mancini and Frantal, 1970; Schürmann, 1972). Morphological evidence for
Fig. 3. A–H: Synaptic electron-lucent and dense core vesicles. A,B: Electron-lucent vesicles immunoreactive for glutamate are round (A) while those immunoreactive for GABA are pleomorphic (B), revealed here in the locust prothoracic ganglion with the postembedding immunogold technique. C–E: In synaptic neuropiles, neuronal profiles immunoreactive for biogenic amines (asterisks) are here revealed using pre-embedding peroxidase immunocytochemistry in the cricket prothoracic ganglion. Processes immunoreactive for octopamine (OA), dopamine (DA), and 5-HT contain clusters of dense core vesicles. In addition, electron-lucent synaptic vesicles associated with conventional dyadic synaptic sites (arrows) may be encountered. F,G: Non-synaptic release of dense core vesicles is commonly observed after potassium stimulation and tannic acid infusion followed by chemical fixation. Membrane fusion of dense core vesicles (arrows) is here seen accompanied by a release of vesicle contents into the extracellular space in cricket brain protocerebral neuropile. H: Omega-figures representing dense core vesicle fusion with the cell membrane are only rarely encountered after conventional fixation of stimulated or unstimulated tissue (Cricket mushroom body a-lobe). Scale bars = 0.2 µm (A,B,H); 1 µm (C–E); 0.5 µm (F,G).
release of the vesicles at such sites has been found in studies of other invertebrates (Günther and Schürmann, 1973).

**Putative Sites of Non-Synaptic Chemical Transmission**

It has been proposed that large diameter dense core vesicles release their contents mainly at sites other than identifiable synaptic active zones (Buma and Roubos, 1986; Golding, 1994; Schürmann et al., 1991). Following conventional aldehyde fixation, however, exo- and endocytotic omega profiles have rarely been seen at extra-synaptic sites in neuropiles where such vesicles are common. Following stimulation of brain tissue with potassium ringer solution containing tannic acid, a dramatic increase in the incidence of non-synaptic omega profiles in these neuropile areas is seen with conventional fixation (Fig. 3F,G). The dimensions of the omega profiles correspond to dense core vesicles that lie free in the cytoplasm (Kerwien and Schürmann 1990). This suggests a paracrine form of release of neuroactive compounds (volume transmission) in insects, in addition to focal synaptic transmission associated with small agranular vesicles (Vizi and Labos, 1991). Volume transmission has been proposed as a feature of the central nervous systems of both vertebrates and invertebrates and is considered to be an early evolutionary development (Golding, 1994). Despite its probable importance, the contribution and functional significance of non-synaptic release of neuroactive compounds from vesicles are largely unknown.

**Electrical Synapses**

In the fly central nervous system, both physiological and ultrastructural evidence has been obtained for electrotonic coupling between identified neurones (Bacon and Strausfeld, 1986; Strausfeld and Bassemir, 1983; Tanouye and Wyman, 1980). Mixed synapses with both the ultrastructural features of chemical and electrotonic connections have also been described (Blagburn et al., 1999). Though less common, electrotonic connections are present between neurones (Siegler, 1982) and between glial cells in the Orthoptera and in other insect groups. Ultrastructural studies of a connection between two visual interneurones in the locust brain (the LGMD and DCMD) have demonstrated that while it is based on thousands of chemical synapses, there are in addition regions of gap-junction like contacts between the two neurones. At these sites, which are typically about 0.1 nm in length, the membranes of the two neurones lie only 2 nm apart (Killmann and Schürmann, 1985). The synapse between the two neurones is a rectifying connection with a high safety factor, but whether its electrophysiological properties justify the conclusion that it has a mixed chemical-electrical nature, is still a matter of debate (Rind, 1984; Rowell and O'Shea, 1980).

Dye coupling between adult and developing insect neurones has been reported for other insects groups (Jacobs et al., 2000; Phelan et al., 1996; Strausfeld and Bassemir, 1983) and has also been observed in the cricket and locust nervous system (Schürmann, personal observation). In Drosophila, studies of electrical connections involving giant fibres have lead to the discovery of a new family of gap junction proteins called innexins, that are structurally distinct from the vertebrate connexins (Phelan et al., 1998). Studies of embryonic gap junctions in the nervous system of Schistocerca have revealed two novel members of the innexin family, G-Inx (1) and G-Inx (2), in neuroblasts and glia that are orthologous to Drosophila innexins (Ganfornina et al., 1999). G-Inx (1) appears to be expressed predominantly in glial elements of the abdominal ganglia. The distribution and dynamic properties of electrical synapses and their contribution to circuitry of the adult locust central nervous system, however, have yet to be determined.

**SYNAPTIC CIRCUITRY**

**Brain Neuropiles and Neurons**

In some prominent regions of the insect brain (e.g., the mushroom bodies, central body complex, and antennal and optic lobes) long known from numerous histological studies, the neuropile is organised into columns, strata, and glomeruli with repeated functional units and with a high degree of obvious geometrical order, which considerably aided analysis of their synaptic circuitry (Leise, 1991; Strausfeld, 1976). Surrounding neuropiles display a structural organisation that is more comparable to that of the ventral cord. Differences in the architecture of these neuropiles can be demonstrated by immunocyt-chemical studies using antibodies against Drosophila synapsin I-protein (Klagges et al., 1996) in insect species and in other invertebrates (Fabian-Fine et al., 1999; Schürmann, unpublished observations). The distribution of Drosophila synapsin I-like immunoreactivity is correlated with clustered synaptic vesicles at synaptic sites and is, therefore, useful in pinpointing synaptic loci in some neuropiles at the light microscopical level (Fig. 4). In neuropiles appearing less geometrically ordered, but that are nevertheless highly structured (the “diffuse neuropiles” of the ventral nerve cord and brain), high levels of synapsin I-like immunoreactivity are quite uniformly distributed. This is in contrast to the neuropiles looking more geometrically structured where the labelling is concentrated in regions that other studies (Schürmann, 1987) have revealed to contain high densities of synaptic vesicles and synaptic sites (Fig. 4D,E).

Within discrete regions of neuropile containing repeated neuronal units, unusually structured synaptic complexes that comprise elements of microcircuitry (divergent, convergent, serial, reciprocal synaptic coupling) are found in both the vertebrate and invertebrate nervous systems (Figs. 1A–E, 4E, 5, 6). The concept of microcircuitry within the insect nervous system is well established (Pearson, 1979) and has received support by the finding of co-distribution of transmitters and neuromodulators in discrete neuropile compartments (see Homberg, pages 189–209, in this issue). Examples of synaptic complexes in Orthopterans studied using electron microscopy, with or without the aid of intracellular neuronal labelling techniques, are found in the mushroom bodies (Iwasaki et al., 1999; Leitch and Laurent, 1996; Mancini and Frontali, 1970; Schürmann, 1974, 1987; Strausfeld and Li, 1999; Trujillo-Cenoz and Melamed, 1962), and in the antennal lobe glomeruli (Boeckh et al., 1970, 1989; Distler, 1990; Distler and Boeckh, 1996). Studies of the optic lobes of
Fig. 4. A–D: Synapsin I-C3 immunocytochemical staining differs in distribution and intensity in different brain neuropilar compartments of the locust (A) and cricket brain (B–D) seen in laser confocal micrographs (A,D) and in conventional fluorescence micrographs (B,C) of vibratome sections. The distribution of immunoreactivity corresponds to vesicle clusters at synaptic sites. A: Note differential staining of strata in the optic lobe neuropiles of the lamina (LA), medulla (ME), and lobula (LO) and immuno-negative parts in the chiasmata (asterisks) and lines perpendicular to strata in the medulla. B,C: Mushroom body compartments of the calyces (CA), peduncle (PE), and α-lobe (α) and the central body part (CB) appear less intensely stained than surrounding neuropiles (asterisk); ocellar neuropiles (triangles) and bridge (BR) are strongly stained. Fluorescence in the neurolemma (star) is non-specific. (Fluorescence micrograph of a horizontal vibratome section through the cricket brain.) D: The pattern of stained boutons in the mushroom body calyx (asterisk) of the cricket is seen in this laser confocal micrograph. E: Similar pre-synaptic boutons (asterisks) in the same calycal neuropile are seen in the electron micrograph. Scale bars = 100 μm (A); 50 μm (B,C); 10 μm (D); 1 μm (E).
Fig. 5. See figure legend on next page.
Orthopterans are relatively rare (O’Carrol et al., 1992). A recent paper describes a special convergent wiring of cholinergic visual interneurons in the locust brain (Rind and Leitinger, 2000; Rind, to appear in Part II of this special issue). In comparison, by far the most intensely investigated insect brain neuroepules are the optic lobes of flies (see Meinertzhagen, 1993).

A deeper understanding of the synaptic circuitry within less geometrically ordered neuropiles is dependent on intracellular staining methods that reveal the structure of single or small numbers of physiologically or morphologically characterised neurones. When carried out in combination with immuno-electron microscopy of neuroactive compounds, this has proved a powerful tool for the structural analysis of synaptic interactions. While the small number of neurones revealed in any one preparation may be seen as a disadvantage, this can be outweighed by the possibility of correlating the physiological properties of a neurone with a knowledge of the nature and distribution of its synaptic interactions.

While such methods have been quite widely applied to neurones in the locust ventral nerve cord (see below), only a few ultrastructural studies have applied these labour intensive techniques to neurones in the brain of the locust and other Orthopterans. Furthermore, the studies so far carried out either aim to describe the nature, distribution, and numbers of synaptic connections in small regions of a neurone’s arborisation or to understand the synaptic wiring of several units in a functional context. No inspection of a single neurone at the ultrastructural level has yet delivered a complete analysis of the range and numbers of input- and output synapses for identified neurones. However, the demonstration of the entire synaptic compliment of single neurones may soon become possible through the refinement of methods of confocal imaging used in combination with immunochemistry. The total number of synapses of single identified neurones has so far only been estimated from selected samples of serial sections or from serial ultrathin sections of restricted parts of their central arborisations. One neurone for which this has been done is the large visual intersegmental interneurone, the descending contralateral movement detector (DCMD). The arborisation of this neurone within the locust brain receives only input synapses that are of two morphologically distinct types and are most abundant on very small diameter branches (Killmann et al., 1999). A total of 8,500 input synapses of different types has been estimated for the whole dendritic tree, among which at least 2,250 contacts arise from the presynaptic lateral giant movement detector (LGMD) neurone. For other identified neurones, estimated total numbers of synaptic contacts vary widely (Killmann et al. 1999; Littlewood and Simmons, 1992; Simmons and Littlewood, 1989). Thus, estimates of the numbers of synaptic contacts that comprise a single physiological connection between a pair of neurones also vary considerably depending on the neurones concerned. For some neurones, the number of contacts constituting a synapse may be as low as a few tens (Meinertzhagen, 1989) but more often this lies in the range of several hundreds if not thousands of synaptic contacts (Burrows et al., 1989; Simmons and Littlewood, 1989; Watson and Burrows, 1983).

**Synaptic Distribution and Circuitry of Single Identified Neurones of the Ventral Nerve Cord**

In the locust ventral nerve cord, representatives of each of the major classes of neurone present have been examined ultrastructurally following intracellular staining with horseradish peroxidase. This has allowed the structure and distribution of their synapses to be described and related to their general physiological properties, and in some cases to their role in the circuitry to which they belong. Comparisons can also be made between neurones within each class and between neurones in different classes.

**Sensory neurones.** The synaptic relationships of a broad range of locust sensory afferent neurones have been investigated. Some supply receptors on the body surface that have a purely exteroceptive role (e.g., prosternal filiform and cercal hairs; Blagburn and Beadle, 1984; Blagburn et al., 1985; Watson, 1990; Watson and Pflüger, 1994), while other surface receptors have a largely proprioceptive role (campaniform sensilla and hair plates; Watson and England, 1991; Watson et al., 1991). The sensory terminals of proprioceptive chordotonal organs that monitor joint movement and position (Altman and Tyrer, 1980; Watson et al., 1993) and of auditory afferent terminals in the cricket (Hardt and Watson, 1999) have also been examined. The central terminals of sensory afferent neurones project somatotopically to modality specific regions of sensory neuropile (Newland, 1991). Most sensory neurones appear to be cholinergic and their terminals, containing round agranular vesicles, make predominantly dyadic output synapses, though there is some evidence that a small proportion may be serotonergic (Lutz and Tyrer, 1988; Parker and Newland, 1985). The central terminals of all of the afferent classes examined receive synaptic input from neuropilar processes that are immunoreactive for GABA (Hardt and Watson, 1999; Watson, 1992; Watson and Pflüger, 1994). Many also receive input from glutamate-immunoreactive processes though the proportion of these inputs varies widely (between 5–50%) depending on the type of afferent neurone (Hardt and Watson, 1999; Watson et al., 1991; Watson and Pflüger, 1984). The release of GABA results in an increase in chloride conductance that induces a presyn-
Fig. 6. See figure legend on next page.
aptic depolarisation of the afferent terminal. This inhibits transmitter release by shunting the currents induced by the action potential as it invades the terminal (Burrows and Laurent, 1993; Hue and Callec, 1983; Watson, 1992). The effects of the glutamatergic inputs are unknown. The only known central glutamatergic synapse in the locust nervous system is one between the fast extensor tibiae and flexor tibiae motorneurones (Parker, 1994; Sombati and Hoyle, 1984). Here glutamate induces a large cation-mediated depolarisation. In the crustacean central nervous system, however, central glutamatergic synapses often induce an increase in chloride conductance (Clarac and Cattaert, 1996; Pearlstein et al., 1998). The input synapses received by the locust sensory afferent terminals are dyadic and where only a single afferent has been labelled, it contributes only one process to the postsynaptic dyad. Where several afferents from a single hair plate were labelled in the same preparation, however, many synapses were seen at which both postsynaptic elements were afferent terminals (Watson et al., 1991). Only two types of hair plate afferent have been identified on physiological criteria, and this configuration would be consistent with many or all of the afferents being gated as a sensory unit. Sensory neurones may also be paired postsynaptically with other classes of neurone. For example, synapses at which a sensory afferent terminal and a sensory interneurone with which it makes synaptic contact elsewhere, form the two postsynaptic elements at a dyadic synapse have been observed (Watson and Pfliiger, unpublished data).

**Efferent Neurones.** Locust muscle is supplied by inhibitory and excitatory motorneurones that induce inhibitory or excitatory postsynaptic potentials at the muscle membrane, and dorsal unpaired median (DUM) modulatory neurones that alter the rate of contraction but have no effect on membrane potential. Each of these classes of neurone has been studied in the electron microscope. The inhibitory neurones are GABAAergic, and there are three pairs in each thoracic ganglion, each neurone supplying many muscles (Watson, 1986). The common inhibitory neurone of the metathoracic ganglion supplies 13 muscles in the proximal part of the leg (Hale and Burrows, 1985) but its central branches contain no synaptic vesicles and it makes no central output synapses (Watson et al., 1985). It receives abundant dyadic input synapses to which it contributes one of the pair of postsynaptic neurites.

The excitatory motor neurones, which are glutamatergic (Bicker et al., 1988; Emson et al., 1974; Watson, 1988; Watson and Seymour-Laurent, 1993), fall into two groups on the basis of their central synaptic connections. Some, such as the slow extensor tibiae and flexor tibiae motorneurones, receive many dyadic input synapses, but make no central output synapses (Watson and Burrows, 1982). Others, such as the tergosternal flight motorneurones and fast extensor tibiae motorneurone (FETi), also make numerous central output synapses (Burrows et al., 1989; Watson, 1984). In most neurones, tracing their neurites over distances of 10–20 μm reveals only simple, one-way synaptic connections with neighbouring neurites (e.g., tergosternal flight motorneurones). The FETi, however, is unique among the arthropod neurones so far studied by serial section reconstruction, in the complexity of the local circuitry in which it participates (Burrows et al., 1989; Watson and Burrows, 1982). Reconstructions of short segments of neurite, or even single ultrathin sections, reveal a complex circuitry involving direct, indirect, and reciprocal connections with other neurones (Fig. 5). The FETi has a dense arborisation of very fine neurites mostly arising directly from the primary neurite, which is quite unlike the sparser more tree-like branching patterns of other motor neurones or interneurones. This may be a consequence of the need to make such complex connections. A considerable proportion of the synapses received by the FETi are monadic (i.e., it is the only postsynaptic element), and this may also reflect the difficulty of establishing and weighting the synapses required for such intricate circuitry within confined volumes of neuropile.

At some of the dyadic synapses to which the FETi contributes the presynaptic component, one of the other neurites involved also contains a presynaptic bar (Fig. 5). The possible significance of these arrangements only becomes apparent if some of the participating neurones can be identified. For example, during the motor programme driving the locust jump, two antagonistic muscles (an extensor and flexor of the tibia) contract at the same time. This is, in part, brought about by a direct synaptic connection between the single fast extensor motor neurone and a number of flexor tibiae motorneurones (Burrows et al., 1989; Hoyle and Burrows, 1973). Direct synaptic connections between the fast extensor and a flexor tibiae motor neurones can be demonstrated when both are labelled by intracellular dye injection (Burrows et al., 1989). Figure 5 shows an example of one of these synapses at which the second, unlabelled postsynaptic process also contains a presynaptic bar. Even where only a conventional dyadic synapse exists between them, a common input onto both occurs within 1 μm in about 85% of the synapses (Fig. 5), thus repeating the pattern in a less rigorous configuration. The identity of the unlabelled third element is unknown but several candidates can be suggested on the basis of known physiological properties of the extensor/flexor connection. An action potential in the extensor tibiae motorneurone causes an excitatory postsynaptic potential or an increase in fir-
ing frequency of the flexor motoneurones (Burrows et al., 1989). In addition, a common drive onto both the extensor and the flexors motoneurones is present that could (though it need not) arise from the unlabelled presynaptic element of the triad. Two identified sources of common input onto the fast extensor and flexor motoneurones are known to be active during the preparatory phase of the jump motor programme. The first comes from a descending mesothoracic interneurone (Pearson and Robertson, 1981) and the second from sensory neurones supplying two campaniform sensilla (cuticular strain detectors) on the proximal tibia of the hind leg (Burrows and Pfüger, 1988).

Another possible role for the unlabelled presynaptic element would be one of modulation of the connection between the extensor and the flexor motoneurone. The static position of the tibia at the time of an action potential in the extensor motoneurone can have a marked effect on the amplitude of the excitatory postsynaptic potential generated in the flexor motoneurone (Heitler and Burrows, 1977a,b). This effect probably arises from sensory input from the femoral chordotonal organ, which monitors the movement and position of the femoro-tibial joint. Because changes in the membrane potential and spike amplitude of the fast extensor motoneurone induced by alterations in joint angle are very small, these could only produce the observed postsynaptic effect if the synapses mediating them were located near the synaptic connection between the fast extensor motoneurone and flexor motoneurones (Jellemé et al., 1997). The observed arrangement of synapses would precisely fulfill this condition. The intricate interconnections of the fast extensor motoneurone revealed by physiological studies, therefore, suggest not just one, but at least three possible roles for the synaptic configurations observed.

The third class of efferent neurone studied in the thoracic ganglia of the locust are octopaminergic dorsal unpaired median (DUM) neurones. At the neuromuscular junction, the axon terminals of these neurones contain numerous large granular vesicles (Hoyle et al., 1974) but though such vesicles are present in small numbers in the bifurcating primary neurites, they are absent from the fine neuropilar branches within the neuropile of the thoracic and abdominal ganglia (Pfüger and Watson, 1995; Watson, 1984). Input synapses received by the DUM neurones are generally dyadic. In preparations where more than one DUM neurone has been labelled, dyadic synapses to which two different DUM neurones each contribute one of the postsynaptic elements can be found (Watson, 1984). Recordings from pairs of DUM neurones in the same ganglion often show a high proportion of common postsynaptic potentials (Duch et al., 1999; Hoyle and Dagan, 1978). Structures that have features of output synapses are only very rarely seen on the postsynaptic element of the triad. Two identified sources of common input onto the presynaptic element of the triad. Two identified sources of common input onto the presynaptic element of the triad. Two identified sources of common input onto the presynaptic element of the triad. Two identified sources of common input onto the presynaptic element of the triad.

Local interneurones. Local interneurones (Fig. 6) have all of their neurites confined within a single ganglion or neuromere or even a particular region within a ganglion. Functionally, they fall into two broad categories, those that produce action potentials and those that do not. Most spiking local interneurones so far studied in the Orthopteran nervous have one set of branches in a sensory neuropile where they receive direct input from sensory afferent neurones (Burrows, 1996). A short unbranching, axon-like neurite may link this field with a second field of branches that lie in a different region of the neuropile and that are predominantly sites of synaptic output. The branches of the input field are often of small diameter and relatively smooth, while those of the output field may be more varicose than those of the input field (Watson and Burrows, 1985). It has been suggested that on the basis of branch morphology alone, the polarity of the neurone and the distribution of input and output synapses can be inferred (Römer and Marquart, 1984; Strausfeld, 1976; Tyrer and Altman, 1974). However, as will be seen in this and other sections of this paper, ultrastructural studies demonstrate that morphology alone is an unreliable guide to synapse distribution. For example, in a population of metathoracic spiking local interneurones receiving sensory input from the leg, about a third of the synapses on the dorsal field of branches that were contacted by sensory afferent neurones were output synapses (Watson and Burrows, 1985). These were particularly concentrated on the larger dorsal neurites, suggesting a degree of compartmentalisation even within a single field of branches. In cricket Omega neurones, however, the input synapses on the branches that receive contacts from auditory afferents outnumber outputs by 10:1 (Watson and Hardt, 1996). In both cases, the identity of the postsynaptic neurones is unknown. The output fields of these two types of interneurone also receive presynaptic input synapses. For the locust metathoracic spiking interneurones, these were outnumbered by the outputs by 4.5:1 and for the Omega neurone by 10:1. Nevertheless, small numbers of input synapses could effectively control the overall output of these neurones.

The synaptic morphology and distribution of non-spiking local interneurones (Fig. 6G) has been studied in both the locust and crayfish ventral nerve cord and the locust brain (Kondoh and Hisada, 1986a,b; Littlewood and Simmons, 1992; Simmons and Littlewood, 1989). In addition, a common drive onto both the extensor and the flexors motoneurones is present that could (though it need not) arise from the unlabelled presynaptic element of the triad. Two identified sources of common input onto the fast extensor and flexor motoneurones are known to be active during the preparatory phase of the jump motor programme. The first comes from a descending mesothoracic interneurone (Pearson and Robertson, 1981) and the second from sensory neurones supplying two campaniform sensilla (cuticular strain detectors) on the proximal tibia of the hind leg (Burrows and Pfüger, 1988).

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Most efferent neurones, regardless of type, make few if any central output synapses. However, those that do may be involved in very complex local circuitry. The extensor tibiae muscle of the locust hind leg is supplied by only two excitatory motoneurones, the FETi whose synaptic properties have just been described, and the slow extensor tibiae motoneurone (SETi) that makes no central output synapses. The FETi is active only in the explosive tibial extension of jumping and kicking, and the SETi only in walking and other slow movements. This distinctiveness in terms of behaviour and circuitry emphasises the uniqueness of individual locust neurones even among those that belong to the same class and contact the same postsynaptic structure.
1989; Watson and Burrows, 1988). Many populations of non-spiking local interneurones appear to have only a single arborisation within the neuropile, and on these branches input and output synapses may be intermingled though inputs are more abundant on smaller diameter branches and outputs on larger ones (Kondoh and Hisada, 1986b). A similar pattern is seen in some local non-spiking interneurones in the locust, while in others, small diameter branches make predominantly output synapses (Watson and Burrows, 1988). One population of crayfish non-spiking interneurones has two distinct arborisations, one of which is composed of smooth branches that received predominantly input synapses, the other being more varicose with intermingled inputs and outputs (Kondo and Hisada, 1986a).

There are, therefore, a variety of patterns of synaptic distribution possible on the branches of these small neurones. Because these neurones function without action potentials, a crucial factor for the way in which they operate is how efficiently potentials can spread through the arborisation. The crayfish neurone with separate input and output arborisations presents one extreme form of organisation. Here the two arborisations are linked by a very large diameter and presumably a low resistance neurite to ensure the efficient spread of potentials between the two fields. In neurones where input and output synapses are intermingled, however, an output synapse will be most strongly affected by the inputs nearest to it. If input synapses from single or functionally equivalent presynaptic and postsynaptic neurones are clustered on particular branches, the interneurone might be functionally compartmentalised. The compartments would not be exclusive, rather inputs from different sources would constitute a series of overlapping spheres of influence having strong effects on nearby output synapses and progressively weaker influences on more distant output sites. This scenario has not been specifically tested, but some of the known physiology of non-spiking interneurones would lend themselves to such an interpretation (Burrows, 1996; Laurent and Burrows, 1989).

Non-spiking neurones can be demonstrated to release neurotransmitter tonically at membrane potentials at which they are typically encountered (Burrows and Siegler, 1978). In the ventral nerve cord of the locust (Watson and Burrows, 1988) and the crayfish (Kondoh and Hisada, 1986a,b), the morphology of the output synapses made by non-spiking interneurones resembles that of synapses seen in spiking interneurones. They are typically dyadic, with a presynaptic bar surrounded by populations of vesicles that are not noticeably more abundant than those in spiking neurones. A different situation prevails, however, in a population of locust brain ocellar L-neurones, which each make both tonic excitatory output synapses onto other L-neurones and phasic inhibitory reciprocal output synapses with each other (Simmons and Littlewood, 1989; Littlewood and Simmons, 1992). Each tonic synapse is composed of some hundreds of dyadic associations at which a presynaptic bar is surrounded by small agranular vesicles. These synapses are characterised by numerous coated omega profiles that may represent sites of membrane re-uptake. By contrast, though the phasic synapses are in many ways similar, the number of dyadic associations and the number of omega profiles at each is lower, perhaps reflecting lower levels of activity.

**Intersegmental Interneurones.** Intersegmental neurones are spiking neurones whose axons link one ganglion to another. They may ascend or descend between two adjacent ganglia or run for considerable distances along the nerve cord arborising in several ganglia and within the brain (Killmann et al., 1999; see Heinrich, to appear in Part II of this special issue). At the light microscope level, examination of the arborisations of these neurones may reveal some fields that are composed of relatively smooth fine branches and others that are composed of varicose branches. These have often been interpreted as being sites of input and output, respectively, but as with the local interneurones, ultrastructural examination usually reveals a more complex pattern that suggests that the neurone may have a variety of functions or modes of operation in different ganglia. Most studies of the synaptic distribution of neurones of this type have examined the arborisations in a single ganglion only, usually the one that contains the cell body of the neurone. One such study is of a locust ascending mesothoracic interneurone that plays a role in flight initiation (Watson and Burrows, 1985). This neurone contains predominantly round agranular vesicles and is probably glutamnergic (Watson and Seymour-Laurent, 1993) and in the mesothoracic ganglion, input and output synapses are present in approximately equal numbers and are intermingled on all branches.

In the cricket prothoracic ganglion, the synaptic distribution of two ascending interneurones, AN1 and AN2, both of which carry auditory information to the brain, have been compared (Hardt and Watson, 1994). The two neurones have quite similar branching patterns in the prothoracic ganglion, but whereas AN1 receives only input synapses, AN2 also makes outputs. Both neurones have many fine branches in the ventral medial auditory neuropile where they receive monosynaptic input from auditory afferents. However, the outputs from AN2 are made only from major branches in this neuropile that are of considerably larger diameter than the equivalent ones in AN1. This pattern of synaptic distribution bears some similarity to that of the input field of the local spiking interneurones though the separation between the fine branches as sites of input and the large diameter ones as sites of output is more extreme in AN2. An intersegmental auditory interneurone in the locust mesothoracic ganglion shows some branches that are input only, while others have both inputs and outputs, with the latter predominating (Peters et al., 1986). The input branches are smooth while those with output are varicose, though as the authors point out, varicosities may be more reliable indicators of clustered mitochondria than synapse sites. AN1 is not the only intersegmental interneurone that has been found to receive only input in its ganglion or region of origin. The same is true of dendrites of the DCMD neurone that lie within the locust brain (Killmann et al., 1999) and of a population of descending intersegmental interneurones that coordinate local locomotor reflexes involving the mesothoracic legs (Watson and Laurent, 1990). This latter population contains both GABAergic and non-GABAergic neurones, which receive direct excita-
tory input from leg mechanoreceptors in the mesothoracic ganglion and inhibitory inputs from local mesothoracic interneurones.

The most complete study so far of synaptic distribution on an Orthopteran intersegmental interneurone has been carried out on the neurone A411 (Watson and Pfüger, 1989). This neurone receives its major input from fields of wind sensitive hairs on the prothorax and head of the locust that terminate in the ventral neuropile of the prothoracic ganglion. The cell body, however, lies in the fourth abdominal ganglion and its axon projects to the brain, arborizing in all of the ganglia in between. Action potentials can be initiated from both the prothoracic and the fourth abdominal ganglia. In the thoracic ganglia, the neurone has fine varicose ventral neurites and much thicker dorsal neurites. In the prothoracic ganglion, the ventral branches receive only input synapses and the dorsal branches make only output. A similar trend is seen in the meso- and metathoracic ganglia, though here some outputs are also made from the ventral branches and some inputs received by the dorsal ones. In the fourth abdominal ganglion, the neurone receives synaptic input only and rostrally running action potentials can be elicited from this ganglion following wind stimulation of the cerci. The effect of these would, however, normally be swamped by the greater sensitivity of the prosternal sensory input that sends action potentials both rostrally and caudally. Physiological studies so far have not revealed the role of the abundant output synapses on the dorsal prothoracic branches though the neurone is known to make direct connections onto mesothoracic flight motorneurones and indirect connections with metathoracic flight steering motorneurones.

**CONCLUSIONS**

In insects, synaptic ultrastructure and synaptic circuitry have been most intensely studied in two groups, the Diptera and the Orthoptera. The fruitfly Drosophila has proved an important model organism for investigations of synapse structure due to its suitability for combined genetic, molecular biological and morphological approaches. Its major disadvantage is the limited accessibility of its neuropiles for combined structural and electrophysiological studies on neuronal connectivity though this has proved possible in other Dipteran species. On the other hand, the studies of identified neurons of locusts and other Orthopterans have delivered by far the most detailed knowledge of physiology in relation to their roles in simple and complex behaviours (Burrows, 1996). The molecular components of the Orthopteran synaptic apparatus have, however, not yet been investigated in detail. The study of Orthopteran synapses is likely to profit directly from the knowledge that has been acquired of the molecules associated with Drosophila synapses and from antibodies raised against them. Further investigations of the functional morphology and role of mature synapses of identified locust neurons should be directed towards three important problems: (1) Understanding the subcellular topography of the synaptic complexes, (2) Investigating the principles of synaptic microcircuitry, (3) Unravelling the pharmacology of identified central synaptic interactions, which has so far received scant attention.

Electron microscopy will remain indispensable for synaptic studies, as it is still the only direct means of unequivocally demonstrating sites of synaptic contact and for localising neurotransmitters and other synapse-associated molecules to the synaptic site. The knowledge of synaptic distribution that is required for the detailed modelling of neuronal interactions is dependent on laborious quantitative electron microscopy. These will most effectively be directed at identified Orthopteran neurones about whose role in central circuitry a wealth of information is already available.

Quantitative computer-aided electron microscopy has demonstrated the existence of two types of synapse in a locust neuron, similar to type 1 and type 2 synapses in vertebrates, which would otherwise be hard to distinguish. If the physiological significance of these differences can be determined, this approach may greatly strengthen the power of ultrastructural analysis. Studies of intracellularly labelled, physiologically characterised neurones have revealed that though dyadic synapses are the most prevalent type of contact between insect neurones, a single neuron may express a mixture of dyadic, monadic, bar-containing, or bar-free synaptic active zones and of subsynaptic densities of variable extent. The functional significance of bars and of the structural variability seen at synapses is still not understood, though some of the implications of dyadic synaptic configurations can be derived from investigations of connections between pairs of identified neurons.

Whereas small electron lucent vesicles in synaptic terminals are assumed to be released from active zone, the sites and consequences of dense core vesicle release remain unclear even though dense core vesicles are found in the majority of nerve fibre profiles. Neuropeptides outnumber classical transmitters by far (see Homberg, pages 189–209, in this issue) and their distribution is concentrated in some brain areas, among them the central body complex. However, in Orthopteran central nervous systems, as well as in other insects and invertebrates, the connectivity and functions of peptidergic interneurones remain largely uninvestigated. These neurons deserve intensified study, as they contribute significantly to local networks. In addition, despite extensive immunocytochemical studies of the Orthopteran nervous system using antibodies raised against most classical transmitters and many insect peptides, the nature of the neurotransmitters used by many populations of interneurones remains completely unknown and until this information is available, a complete understanding of the principles underlying insect neuronal circuitry will not be possible.

Despite the attention that has been devoted to investigations of the Orthopteran central nervous systems, our knowledge of their neural circuits is still fragmentary. Numerous light microscopical studies have provided a useful framework for the understanding of the different neuropilar compartments of the insect brain, revealing the major classes of nerve cells present and many common underlying architectural principles. Inevitably, these studies have been directed at circuitry that is particularly amenable for study and consequently much remains to be discovered. For the fly brain, a considerable amount of valuable data has been
obtained from studies of the optic lobe neuropiles (Meinertzhagen 1989; Strausfeld, 1976), while in Orthopteran brains the antennal lobes and mushroom bodies have been fertile ground for studies of olfactory information processing, learning, and memory. A deeper knowledge of synaptic structure and principles of circuitry, and their structural and functional dynamics, will be gained by further intense studies from these highly organised neuropiles, whereas the investigations of single identified neurons of all types are indispensable for a more profound understanding the design principles of circuitry expressed in less structurally organised brain neuropiles and the ventral cord.

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