Thoracic Interneurons, Motorneurons and Sensory Neurons of *Locusta Migratoria* (Insecta: Orthoptera) in Primary Cell Culture

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Abstract: The aim of this study was to establish a cell culture system with identified classes of locust neurons (interneurons, motorneurons and sensory neurons). The cells belonging to the different classes were distinguished in cell culture by vital dyes, which had been applied to the neurons *in situ*. From the various dyes tested fluorescent marked dextrans (10.000MW) gave the best results. The cells survived for up to 28 days in culture and approx. half of the cells grew processes, except of the sensory neurons, which never formed any processes. The different neurons were comparatively investigated, e.g. with immunhistochemistry: 86% of motorneuron were glutamate immunoreactive and 50% of the interneurons exhibited GABA-like immunoreactivity. The cells had resting potentials between -20 and -60mV and did not show spontanous action potentials. Action potentials could be elicited by current injection in 8% of interneurons and 26% of motorneurons, but not in sensory neurons. The vital marking of cells allowed to study distinct neurons in cell culture and to compare their morphology and physiology.

INTRODUCTION

In vertebrates and invertebrates cell cultures have been prooven to be an important tool to address developmental questions as well as those for their physiology, like the function of single ion channels [1]. Since many years also insect neurons have been used in cell culture [2-9]. The cells in those cultures are often unidentified, thereby loosing the advantages of the identified neuron concept. In insects, the concept of the individually identifiable neuron has been very successful and revealed many details of the development and wiring of neuronal networks. Although aspects of the development and the physiology of many of such neurons within the networks are known, numerous details are still unknown. A cell culture system can provide a perfect tool for physiological analysis, given that distinct neurons can be studied under comparable conditions. In selected cases it has been possible to study single types of neurons, like the medial neurosecretory cells [10, 11], the dorsal unpaired median neurons [12] and giant interneurons [13]. In *Manduca sexta* identified motorneurons could be studied together with sensory neurons [8]. In locusts, one of the insects in which neuronal networks are intensively analysed, such a specific cell culture for different classes of neurons is not established. Most studies involved a mixture of different unidentified neurons of the central nervous system [7].

Here we report a method for studying distinct classes of insect neurons in locusts by labelling motorneurons, interneurons and sensory neurons prior to dissociation. We compared their survival correlated to different vital dyes. Furthermore, we present first results on the growth, immunhistochemistry and physiology of the different classes of neurons.

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MATERIALS AND METHODS

All investigations were performed with neurons obtained from fifth instar larvae of *Locusta migratoria*. Animals of both sexes were taken from the colony of the I. Zoological Institute, Göttingen. Experimental animals were dipped for 10 sec into 70% ethanol and subsequently attached to a plasticine holder. Thereafter, the sternum of the meso- and metathorax was opened and the meso- and metathoracic ganglia were exposed. To identify different classes of neurons *in vitro* these neurons had to be labelled with a vital dye *in situ* prior to dissociation. Tested dyes were aquateous solutions (5%) of Lucifer Yellow, Texas Red (Sigma), TRITC- and FITC-coupled dextranes of 3,000D, 10,000D, and 70,000D molecular weight and 1,1´-dioctadecyl-3,3,3´3-tetramethylindocarbocyanine (DiI)-cristals (Molecular Probes). Each marker was used in at least 20 preparations. For labelling ascending interneurons, the connectives between meso- and prothoracic ganglion were cut. The caudal part of one connective was placed within a capillary filled with an aquateous dye solution. The preparation was put in a moist chamber at 4°C for 24h. Motorneurons were labelled by centripetal backfilling of the leg nerve N5 of the metathoracic ganglion. After the diffusion of the dye, the meso- and metathoracic ganglion (for interneurons) or the metathoracic ganglion alone (for motorneurons) were removed and placed in a petri dish filled with Leibovitz L-15 medium (InVitrogen). Subsequently, the ganglia were incubated in a solution of L-15 supplemented with 3mg collagenase/dispase per ml (Boehringer) for 40min at room temperature. The reaction was stopped by fresh L-15 medium supplemented with 5% foetal calf serum (FCS, Sigma). The ganglia were transfered to a petri dish coated with Concavalin A (Boehringer) and the cells were mechanically dissociated. Remaining parts of the ganglion were removed and all cells were allowed to settle on the bottom of the petri dish. The cells were incubated at 25°C and the medium (L-15 with 5% FCS) was changed every seven days. In cases where central neurons

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without a specific labelling were studied, the meso- and metathoracic ganglia were removed from the animal directly after opening the ventral cuticle.

For sensory neurons a different preparation was used. Animals were laterally attached to a holder and the Müller`s organ was removed. This sensory ganglion contains the somata of the scolopidial cells of the ear. It was placed into a petri dish filled with a solution of L-15 and Hanks-medium (1:1; Invitrogen; [14]). A dye deposit surrounded by petroleum jelly was formed in the center of the dish. The Müller`s organ was placed near the deposit and the stump of the tympanal nerve was inserted into the dye solution. Alternatively, a DiI cristal was placed onto the nerve stump. After 24h at 4°C the organ was placed in calcium free Hanks medium, supplemented with 3mg collagenase/dispase per ml for 15min followed by an incubation in L-15 with 0.5mg trypsin /ml. Four to six Müller`s organs were transfered to a concavalin coated petri dish and the cells were mechanically dissociated.

The cell cultures were photographed on an inverted microscope (Leica DM IL, with Wild MPS 48/52) to document their survival and growth. The size of soma and maximal extensions of cell processes were measured from these photos after calibration. The photos were scanned and arranged using Adobe Photoshop.

Immunhistochemistry

To characterize the labelled neurons further immunhistochemistry for neurotransmitters was performed. Since the dye was barely detectable within the marked neurons after PAP/DAB immunhistochemistry, the positions of labelled cells (motorneurons, interneurons) were indicated by scratches into the bottom of the petri dish. The cells were fixed for 10min with a mixture of glutaraldehyde, picric acid and acetic acid [15]. After several washes with 0.1M Tris buffer (pH 7.3) with 0.1% Triton X-100, non-specific staining was blocked by 0.5% non-immune goat serum (Sigma) in Tris buffer. Subsequently, the cells were incubated in a primary antibody solution of either anti-glutamate (1:1,500) or anti-GABA (gamma-aminobutyric acid; 1:4,000; both antisera from Arnel, New York) for 2h at 4°C. After three washes with buffer, the cells were incubated with goat-anti-rabbit solution (1:100; Sigma) for 45min at room temperature. An incubation with peroxidase-anti-peroxidase complex solution (1:800; Sigma) followed for 1h at room temperature. Thereafter, a marking reaction was performed with a DAB/H_2O_2 kit (Vector laboratories) [15] and the marked cells were counted.

Additionally, immunhistochemical marking with anti-horseradish peroxidase (anti-HRP) was performed. Anti-HRP binds specifically to an epitope on insect neurons [16] and can be used to reveal neuronal character and structure of the cells in culture. The procedure was the same as described above except that cells were fixed with 4% paraformaldehyde and the concentration of anti-HRP (Sigma) was 1: 800.

Electrophysiology

Electrical potentials of the neurons were registered with intracellular recordings from the cell body at room temperature. Glass microelectrodes with 80-120 megaohm resistance filled with potassiumacetate (1M) were inserted into the marked cell under visual control. The membrane potential was registered as drop of the potential while the electrode was driven into the cell. Depolarizing current was injected to elicit action potentials. The potentials were recorded on magnetic tape and the recordings were analysed with Turbolab 5.0 (Stemmer software) and Neurolab [17]. To reveal neuronal morphology some cells were injected with Lucifer Yellow (Sigma). Therefore the tip of glass capillary was filled with 5% Lucifer Yellow and the dye was iontophoretically injected into the cell with hyperpolarizing current (1-3nA).

RESULTS

1. Labelling of Neurons and their Survival

Different classes of neurons were labelled by application of retrogradly transported dyes to the cut axons before dissociation for cell culture. Thoracic ascending interneurons were labelled *via* the connective between the pro- and the mesothoracic ganglion, hindleg motorneurons *via* the nerve N5 of the metathoracic ganglion and auditory sensory neurons *via* the tympanal nerve. The somata of the labelled interneurons were located mostly in the mesothoracic ganglion (Fig. **1b**), but also within the metathoracic ganglion. In total, 45-55 cell bodies could be distinguished in each preparation. Since usually the connectives on both sides of the animal were labelled at most 90-110 marked cells could be expected

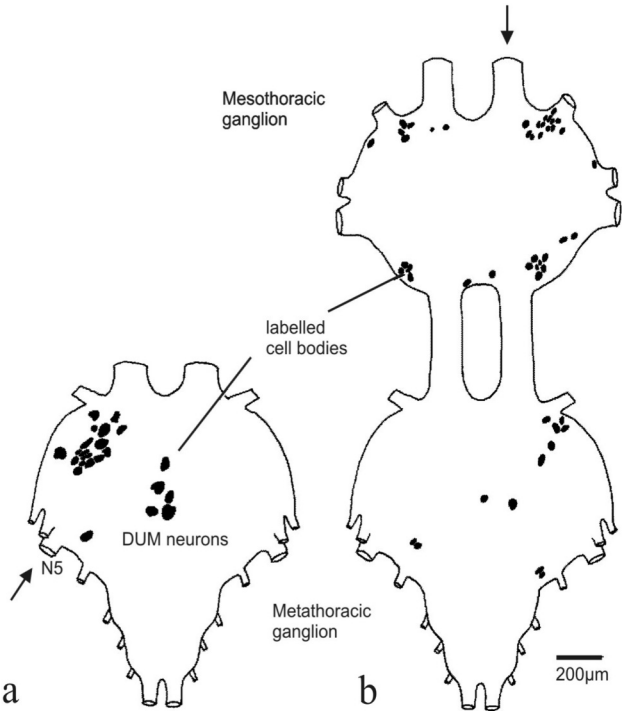

Fig. (1). Semischematic drawing of the meso- and metathoracic ganglia of larval *Locusta migratoria* with labelled cell bodies. (**a**) Motorneuronal cell bodies were labelled by applying dye to the hind leg nerve (N5, arrow). Most cell bodies are located laterally; some somata were in a median dorsal position (DUM neurons) (**b**) Cell bodies of ascending interneurons were marked from the rostral connective of the mesothoracic ganglion (arrow). The cell bodies are found in distinct clusters in both ganglia; shown are the clusters for labelling of one nerve or one connective. By contrast for cell culture usually two nerves or two connectives have been labeled.

in the culture dish. In some experiments 85 of the ascending interneurons were detected in the culture dish.

The cell bodies of the marked motorneurons were located only within the metathoracic ganglion (Figs. **1a**, **2a**). 18-25 somata were labelled *via* a leg nerve, usually including 5 dorsal unpaired neurons (DUM-neurons), whose axons extend bilaterally in both leg nerves. Again, the nerves of both sides were labelled, resulting in 31-45 labelled neurons per ganglion. Within a petri dish up to 21 marked cell bodies could be found.

Within the Müller`s organ which contains the sensory cells of the ear, about 40 receptor cells could be labelled in situ (Fig. **2b**). About 7 marked cells per sensory ganglion were recovered in a petri dish.

The choice of the marker was crucial for easy and repetitive identification of the cells, their growth and survival. About 20% of the unlabelled neurons died within the first 24h *in vitro* (Fig. **3a**). The mortality rate decreased subsequently and after 14 days 50% of the central neurons were still alive. The dyes reduced the survival rate of the neurons. When ascending interneurons and motorneurons were labelled with Lucifer Yellow or Texas Red most cells died within the first two days (Fig. **3b**; n= 378 and n=166 cells, respectively). No Texas Red labelled cells were found after 6 days in culture. The survival was better for cells labelled with fluorescent dextrans. After two days in culture 39% of cells labelled with Dextran 3.000D (n=154) and 58% of cells labelled with Dextran 10.000D (n=86) were still alive. At day 8 the percentage of surviving cells dropped below 20%, but by that time the cells had been illuminated at least 4 times (on each of the test days). The best results were achieved with Dextran with a molecular weight of 10,000D and all further labelling was performed with this dye. The survival of marked cells is reduced after the first week in culture in comparison to the unlabelled controls (Fig. **3c**). The survival of ascending interneurons and motorneurons was very similar; both classes of central neurons survived much better than labelled sensory neurons (Fig. **3c**).

2. Morphology and Immunocytochemistry of the Different Classes of Neurons

After marking the different classes of cells with dextrans, they could be distinguished in primary cell culture from cells of unknown origin by their fluorescence (Figs. **2c**,**d**). Although unlabelled cells with spherical cell bodies and extended processes could be identified by anti-horseradish peroxidase (anti-HRP) immunostaining as neurons (Fig. **2g**), some uncertainty about the cell type remain during analysis. Most of the following descriptions therefore are based only

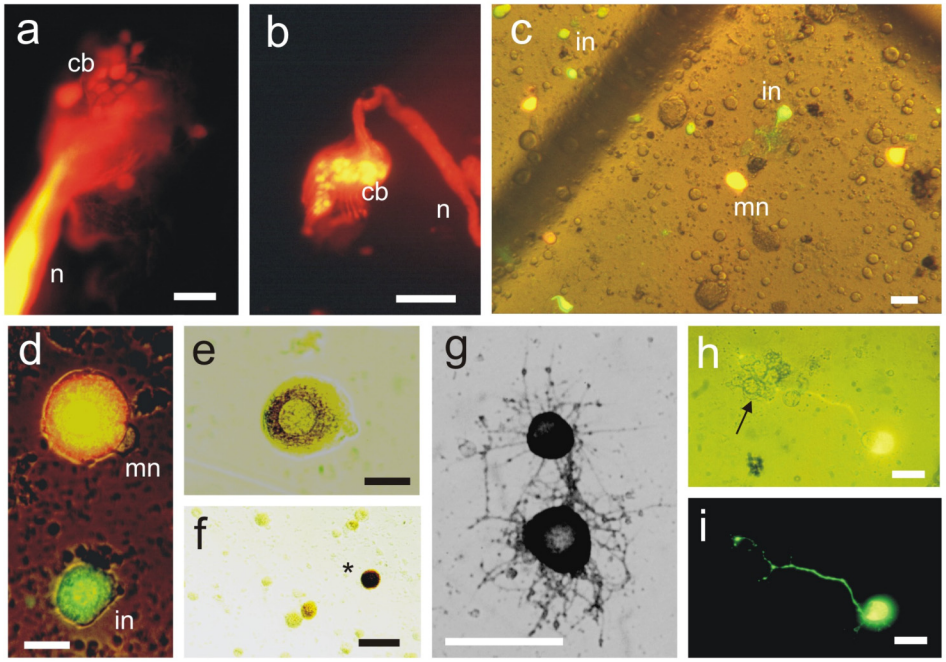

Fig. (2). Photoplate of in situ labellings and of neurons in primary cell culture. (**a**) Labelled cell bodies (cb) and their diffuse arborization pattern of leg motorneurons in the ipsilateral half of the metathoracic ganglion. N= nerve used for the backfill with TRITC-coupled Dextran MW 10.000D. (**b**) Sensory neurons within the Müller`s organ retrogradly labelled with TRITC-coupled Dextran MW 10.000D. The cell bodies (cb) are orange because of accumulated dye whereas the axons within the tympanal nerve (n) and the dendrites are of red colour. (**c**) Dissociated cells after 3 days in primary cell culture. The cells were prepared from 2 ganglia with marked interneurons (in; green; FITC-coupled Dextran MW 10.000D and 2 ganglia with marked motorneurons (mn; orange, TRITC-coupled Dextran MW 10.000D). (**d**) Co-culture of a marked interneuron (in) and motorneuron (mn) in close distance after one day in culture. (**e**) A glutamate-immunoreactive cell body of a motorneuron after 3 days in culture. (**f**) Interneurons of the meso- and metathoracic ganglion stained with anti-GABA after 7 days in culture. The right cell (asterix) is marked and GABA-immunoreactive, whereas the other cells were not marked. (**g**) Two cells of the metathoracic ganglion showing multipolar morphology and overlapping processes. The cells were 14 days in culture and were stained with anti-HRP. (**h, i**) A unipolar interneuron injected with Lucifer Yellow after 14 days in culture. The neuron extends a process to a neighboring cell group (arrow in h), where it arborizes. **h** shows the cells culture with backlight and (**i**) with epi-fluorescence. Scale bars: **a**,**b** 100 µm, **c,d,e,h, i** 20 µm, **f,g** 50µm.

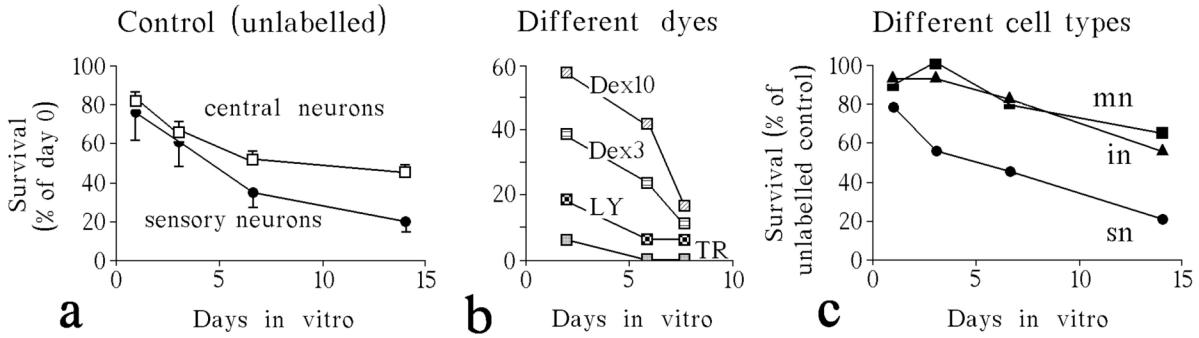

Fig. (3). Survival of cells in primary cell culture. (**a**) Unlabelled neurons of the central nervous system (central neurons, n=1336 cells at day 0) and sensory neurons (n=63) of the Müller`s organ. (**b**) Ascending interneurons and motorneurons were labelled with different dyes and their data pooled. Dex3, Dex10: FITC- or TRITC-coupled dextrans of 3,000D or 10,000D molecular weight (n=154 cells at day 0 and n=86, respectively); LY: Lucifer Yellow (n=378); TR: Texas Red (n=166). (**c**) Survival of ascending interneurons (in; n=287 cells at day 0), motorneurons (mn; n=45) and sensory neurons (sn; n=26) labelled with FITC- or TRITC-coupled dextran (10,000D) as percentage of the unlabelled central neurons or sensory neurons.

on labelled cells. The somata of ascending interneurons usually had a spherical shape and ranged between 20 and 40 µm in diameter (Fig. **4a**). About half of the cells showed a growth of neurites within the first 24h (Fig. **4b**). The percentage increased to about 70% of all living interneurons after 14 days in culture. The cells were often multipolar with processes growing from numerous positions around the cell bodies (Fig. **2g**). In cases where the primary neurite was still attached to the soma after dissociation a growth from the tip of the neurite could be observed. The number of processes and their maximal distance from the soma increased steadily during the incubation period (Figs. **4c**,**d**). Injections with Lucifer Yellow revealed the processes of single neurons (Figs. **2h**, **i**). The marked interneuron shown in Fig. (**2i**) extended a process to a neighboring cell group (arrow in Fig. **2h**).

Cell bodies of motorneurons had the same spherical shape as those of interneurons although their diameter is larger (Fig. **4a**). After 14 days in culture the mean diameter was as large as 60 µm. In some cases the growth of single somata was followed to confirm that the individual cell bodies grew in size. The percentage of motorneurons with processes ranged between 42% and 67% (Fig. **4b**). They had usually a limited number of processes (1 to 3; Fig. **4d**), although in some cases a multipolar morphology with 9 processes could be observed. In single cases it could be observed that grown processes were reduced later.

Cell bodies of labelled sensory neurons had a diameter between 6 and 11 µm (Fig. **4a**) and were of spherical or elliptical shape. The neurons usually did not change their shape nor did they grow processes. However, cells that were labelled after 14 days still had a viable appearance. Another

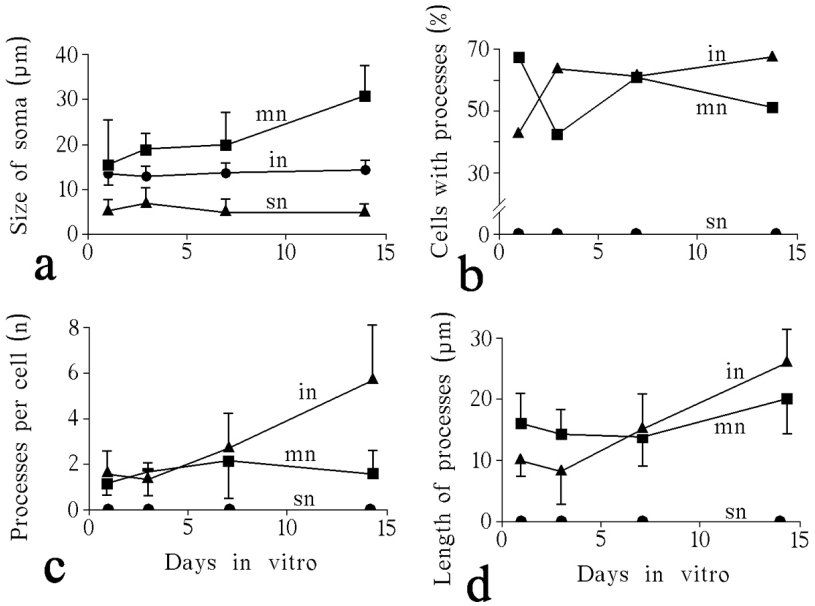

Fig. (4). Morphological parameters of the different classes of neurons in cell culture. (**a**) The size (diameter) of the marked cell bodies. (**b**) Cells with processes as a percentage of all marked cells. (**c**) The number of processes per cell of all marked neurons which had grown processes. (**d**) The length of processes. The length was measured as the maximal distance of the process tip to the cell body. in= interneurons, mn= motorneurons, sn= sensory neurons; n=7-36 in **a**, **b** and n=7-17 in **c**, **d**.

indication that the cells were alive came from the results that a growth of processes could be archived, when the medium was supplemented with certain vertebrate growth factors [18].

We tested labelled and unlabelled cells of the central nervous system for their immunoreactivity to two neurotransmitters, gamma-aminobutyric acid (GABA) and glutamate, both are known to occur within the central neurons system [19, 20]. Within a cell culture of unmarked cells of the thoracic ganglia approx. 15% of all prospective neurons were immunoreactive for anti-glutamate and approx. 10% of these cells for anti-GABA (data not shown). Since glutamate is known as excitatory neurotransmitter in many motorneurons [19], and GABA as neurotransmitter in many interneurons [20], we tested marked cells for their glutamate and GABA immunoreactivity, respectively. 86% of marked motorneurons (n=28) showed glutamate immunoreactivity at day 1 in cell culture (Fig. **2e**). The percentage of anti-GABA positive interneurons after one day in culture was 50% (n=24; Fig. **2f**).

3. Electrophysiology

Neurons of all three classes were investigated electrophysiologically with intracellular recordings. At first the resting potential of the cells was measured in dependence of the time in culture and the class of cell. Sensory neurons had the least negative resting potential ranging between -20mV and -40mV, ascending interneurons ranged from -20mV to -60mV and motorneurons ranged from -20mV to -100mV. The resting potential of both classes of neurons from the central nervous system cells did not vary with time (data not shown).

We did not observe any spontaneous firing of the cells, but action potentials could be elicited with depolarizing current injections into cultured cells. This was possible in 26% of recorded motorneurons (n=23), in 8% of the interneurons (2 out of 22) and in none of the sensory neurons (n=25; Fig. **5**). The thresholds for firing of actionpotentials varied between 1.6 - 2.0nA of depolarizing current for motorneurons. Both interneurons required higher current injection, 2.5 and 9.5nA, respectively, before firing could be elicited. The action potentials of the central cells had an amplitude between 42 - 110mV and a duration between 0.6 - 2.4ms. Usually, the latency decreased and the frequency of action potentials increased with increasing depolarization (Figs. **5a-c**). Only one motorneuron did not show this correlation. This neuron fired with maximum frequency (350Hz within the phasic burst) above a threshold of 1.8nA (Fig. **5b**). Further increase of current injection did not change the frequency. Sensory neurons never showed action potentials, despite injection of large currents (Fig. **5d**).

DISCUSSION

In this study different classes of identified neurons of *Locusta migratoria* were investigated in a primary cell culture. For discrimination between interneurons, motorneurons

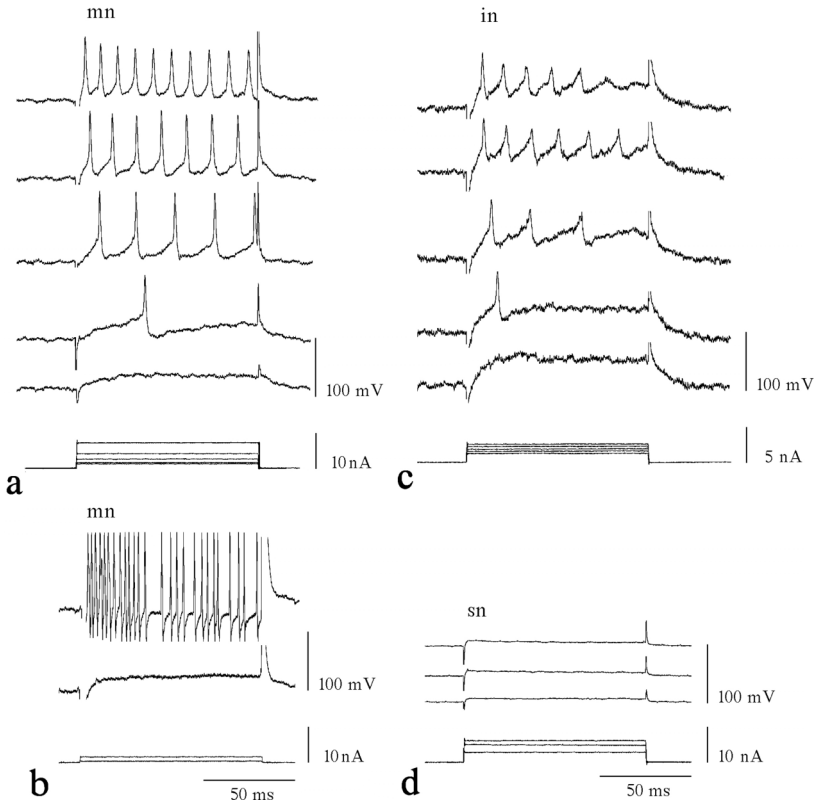

Fig. (5). Intracellular recordings of four neurons with their responses to depolarizing current injections. (**a**) A motorneuron (mn) after 3 days in cell culture. The frequency of action potentials increased with increasing current injections. The threshold was 1.7 nA. (**b**) Another motorneuron after 3 days in cell culture showing a different firing pattern. Current injection above the threshold of 1.8 nA released a strong burst of action potentials which was not altered by higher currents. (**c**) An interneuron (in) after 4 days in culture. Above a threshold of 2.8 nA action potentials were elicited. (**d**) In sensory neurons (sn) no action potential could be elicited.

and sensory cells within the culture dish the neurons have been marked with fluorescent dyes *in situ*. This allows an unequivocal identification in the petri dish. *In vitro* the neurons of the three classes showed different morphological and physiological characteristics. In the following, we discuss firstly methodological aspects and secondly differences between the classes of neurons *in vitro*.

Method

The used cell culture method yielded reliable and repeatable results. The chosen parameters proofed to be the best in a test series using other coating (poly-lysine), other media (Grace medium, Schneiders medium, TC-100) or other fetal calf serum concentrations (data not shown). Comparable parameters have also been used for antennal sensory neurons of Manduca [21] or of the central nervous system of locusts [7].

The choice of the dye for marking the neurons was crucial for easy and repetitive identification of the cells, reliable growth and survival for at least 14 days in culture. Marker substances like horseradishperoxidase or Neurobiotin could easily be applied to the neurons, but methods of visualization of marked cells were not practicable for investigations on living cells. Therefore fluorescent dyes were used like Lucifer Yellow and Texas Red. These dyes could be easily applied to the neurons and they allowed visualization of the cells in culture. However, the marked cells had a very poor survival rate, possibly linked to the production of cytotoxic radicals during illumination. Other fluorescent dyes were obviously less cytotoxic. The carbocyanine dye DiI has been used successfully for identification of motorneurons of *Manduca sexta* in cell culture [22, 23]. We found the hydrophobic DiI less practicable to apply to the stump of a nerve or of a connective. The cristal often was washed away while trying to keep the nerve covered by medium. Therefore, dextrans proofed to be best for labelling *in situ* and cell survival *in vitro*. Dextrans are availiable coupled to different fluorochromes and in different sizes. We found no difference in the survival whether FITC- or TRITC-coupled dextrans were used (data not shown). This finding is in agreement with results obtained in frogs [24]. Of the dextrans tested, those with a molecular weight of 3.000D and 10.000D did successful label cells. However, the percentage of cells marked with dextran 3.000D decreased more rapidly than those of cells marked with dextran 10.000D. The intensity of fluorescence of cells marked with dextran 3.000D seemed also to decrease, perhaps indicating a leakage out of the cell. Dextran with a molecular weight of 70.000D did not label the cells, which is probably due to a lack of axonal transport, again reported also for frog neurons [24]. When repetitively visualized the survival rate of dextran (10,000D) labelled cells dropped to about 20% after 8 days in culture. The survival rate was better when the culture dishes were only checked on the first day (to ensure the presence of labelled cells) and on the day of experiment. Thus, the dextran 10.000D proofed to be best suited for easy and repetitive identification of cells for 14 days in culture. Injection of similar tracer substances into a neuropile could also be used to identify sensory interneurons in primary cell culture [25].

The yield of labelled neurons could be quite high, varying from 18% to 85%. The best yield was obtained for interneurons which have a relatively high number of labelled cells per ganglion and showed a good survival during the dissociation process. The yield of motorneurons was lower, which might be related to the size of the cell bodies, as larger cells are more easily destroyed during dissociation (titration with small diameter pipette). The lowest yield has been observed for the sensory cells, which can be explained by the small size of the organ, which makes handling for the dissociation of single cells difficult. Furthermore the glia sheat of the sensory ganglion seems to be rather strong and tight. The small size also might be responsible for the incomplete labelling of sensory cells (about 40 cells from 60-80 receptor cells per Müller`s organ [26]). The yield of cells for the primary culture was still much better than in other studies on dissociated scolopidial sensory neurons (5-30 out of 2000 cells [14]). Nevertheless, mechanosensory neurons had been investigated physiologically in primary cell culture [27].

Comparison of the Different Classes of Neurons

Growth and Morphology

All marked cells bodies had a spherical shape. Thus, it can be deduced that this shape is typically for neurons in cell culture. Flat cells of irregular shape and varying sizes represented a second morphological type and were probably glial cells. The marked cells of the different classes of neurons had different diameters of their somata. Interneurons were smaller than motorneurons, which is in agreement with the *in situ* situation. Altogether, the diameters were similar to those measured by Kirchhof and Bicker [7] for thoracic neurons of *Locusta migratoria*. The peripheral sensory cells of the auditory organ were much smaller than the central neurons. Cultured sensory cells of other insects have different sizes, 4 µm in olfactory sensory cells of *Manduca* [28], 7-15 µm in the antenna of the blowfly [29] and 20 µm in chordotonal cells of cockroaches [14].

Interestingly, the soma diameter of motorneurons increased with time in cell culture. For some individual motorneurons it could be observed that they grew and then died. Therefore, it is possible that a growth regulation mechanism is disturbed in cell culture. This increase in cell soma diameter could be stopped by the addition of the vertebrate neurotrophic factor GNDF (glia cell line derived neurotrophic factor; [18]).

The growth of neurites was very similar for interneurons and motorneurons, but not for the sensory neurons (see below). In inter- and motorneurons only a certain percentage of the cells formed processes, usually thin filopodial like neurites. Therefore, the cells had a multipolar appearance, which is typical for cells in culture but not for the *in situ* morphology, (e.g. compare [30] for auditory interneurons or [31] for motorneurons). An *in situ*-like morphology of cultured cells has only rarely been reported. Dorsal unpaired median neurons (DUM neurons) sometimes showed typical branching patterns in culture [32], but in other studies this was not confirmed [12]. If a primary neurite was still attached to the cell soma after dissociation only one larger process extended from the stump of the neurite. This had also been observed in other studies [10]. The primary neurite was not absorbed into the cell, which may be due to the strong adhesion to the substrate [12, 33]. Filopodia were also attached very strongly to the substrate which became obvious in immunostaining ex-

periments. During the washing procedure sometimes the soma was removed leaving the cell processes attached to the substrate. No difference in the formation of the processes could be detected between interneurons and motorneurons within the first week of cell culture. After 14 days in culture, the interneurons had formed many more processes than motorneurons and exhibited usually a clear multipolar morphology. The length of processes was similar in both populations. In both cases they reached a mean of 40µm measured as distance from the cell soma, which is much less than in situ. The processes might extend further in a haemolymph conditoned medium [7].

Immunoreactivity

It could be shown that glutamate-immunoreactivity was found in excitatory motorneurons which use glutamate as transmitter [19]. GABA-immunoreactivity was found in local and multisegmental interneurons [20, 34], while ascending interneurons have not been tested explicitly. Our results show that the neurons maintain their immunreactivity in primary cell culture, because many motorneurons are glutamate immunoreactive and many interneurons show a GABA-like immunoreactivity.

Physiology

For physiology experiments only cells with a healthy morphological appearance were used. Further criteria for healthy cells were a drop of the potential as the microelectrode penetrated the cell membrane and changes in the potential during depolarization (except for sensory cells). In some cases the cells were subsequently filled with Lucifer Yellow to visualize their morphology and to ensure that no leakage occured.

The measured resting potentials ranged from -20 to -60mV. The same range has been reported by [5] for locust nymphal neurons. In neurons of cockroaches the measured resting potentials varied from -50mV to -60mV [12, 35]. The membrane potentials of cells *in vitro* are similar to those measured *in situ*, although they tend to be less negative. For the hindleg motoneurons resting potentials of -45 to -60mV have been reported [36]. We observed similar potentials in cell culture. We could not record spontaneously evoked action potentials, but they could be elicited by depolarizing current injection in a certain percentage of the investigated cells. The percentage was higher in motorneurons (26%) than in interneurons (8%). Since the cell somata of insect neurons are *in situ* usually inexcitable [36] the low percentage is not surprising. Accordingly, no action potentials have been found in thoracic neurons of cockroaches, except in DUM-neurons, which are known to be excitable *in situ* [12]. Some of the motorneurons which showed action potentials in our study might have been DUM neurons. However, the situation is less clear since in other studies action potentials recorded from neuronal somata have been reported. Especially, neurosecretory cells exhibit spontaneous activity [10, 37]. But also other cells like embryonic cells of cockroaches might develop overshooting action potentials in cell culture [35].

Sensory neurons of insects have rarely been taken in cell culture. The best investigated receptor neurons are olfactory receptor neurons of the antennae of male *Manduca sexta* [38-40]. On mechanosensory neurons of cockroaches patch clamp experiments on chloride channels have been performed [14]. Mechanosensitive channels could be detected in cultured neurons of Manduca sexta [27]. So far it could only be speculated why the auditory sensory cells of the locust did not show any action potentials. The resting potentials were with -30mV just within the range reported in an *in situ* study (-30 mV to -60 mV [41]). Further studies are needed to show which ion channels are present in the soma of these sensory cells.

We have been able to to establish a cell culture system in which different classes of locust neurons (interneurons, motorneurons and sensory neurons) can be distinguished. After in situ labelling with vital dyes the neurons survived and exhibited different properties in a primary cell culture.

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REFERENCES

[1] Cestelli A, Savettieri G, Salemi G, Di Liego I. Neuronal Cell Cultures: A Tool for Investigations in Developmental Neurobiology. Neurochem Res 1992; 17: 1163-1180.

[2] Provine RR, Aloe L, Seshan KR. Spontaneous bioelectric activity in long term cultures of the embryonic insect central nervous system. Brain Res 1973; 56: 364-370.

[3] Beadle DJ, Hicks D, Middleton C. Fine structure of *Periplaneta americana* neurons in long-term culture. J Neurocytol 1982; 11: 611-626.

[4] Beadle DJ, Botham RP, Lees G. Acetylcholine receptors on cultured insect neurones. J Physiol 1983; 340: 59-60.

[5] Giles DP, Usherwood PNR. Locust nymphal neurones in culture: a new technique for studying the physiology and pharmacology of insect central neurones. Comp Biochem Physiol 1985; 80C: 53-59.

[6] Hicks D, Beadle DJ, Giles DP, Usherwood PNR. Ultrastructure of dissociated nerve cells of *Periplaneta americana* (L.) (Dictyoptera: Blattidae) growing in culture. Int J Ins Morphol Embryol 1981; 10: 225-233.

[7] Kirchhof B, Bicker G. Growth properties of larval and adult Locust neurons in primary cell culture. J Comp Neurol 1992; 323: 411-422.

[8] Melville JM, Hoffman, KL, Jarrard JE, Weeks JC. Cell culture of mechanoreceptor neurons innervating proleg sensory hairs in *Manduca sexta* larvae, and co-culture with target motoneurons. Cell Tissue Res 2003; 311: 117-130.

[9] Vomel M, Wegener C. Neurotransmitter induced changes in the intracellular calcium concentration suggest a differential central modulation of CCAP neuron subsets in Drosophila. Dev Neurobiol 2007; 67: 792-808.

[10] Rössler W, Bickmeyer U. Locust medial neurosecretory cells *in vitro*: morphology, electrophysiological properties and effects of temperature. J Exp Biol 1993; 183: 323-339.

[11] Bickmeyer U, Rössler W, Wiegand H. Calcium channel currents in cultured Pars intercerebralis neurosecretory cells of adult *Locusta migratoria*. J Exp Biol 1994; 197: 393-398.

[12] Newland PL, Smith PJS, Howes EA. Regenerating adult cockroach dorsal unpaired median neurones *in vitro* retain their *in vivo* membrane characteristics. J Exp Biol 1993; 179: 323-329.

[13] Kloppenburg P, Hörner M. Voltage-activated currents in identified giant interneurons isolated from adult crickets *Gryllus bimaculatus*. J Exp Biol 1998; 201: 2529-2541.

[14] Stockbridge LL, French AS, Sanders EJ. Dissociation and culture of mechanosensory neurons for patch clamp analysis. Brain Res 1990; 523: 161-166.

[15] Lins F, Lakes-Harlan R. Interneurons with inhibitory effects on stridulation in grasshoppers exhibit GABA-like immunoreactivity. Brain Res 1994; 635: 103-112.

[16] Jan LY, Jan YN. Antibodies to horseradish peroxidase as specific neuronal markers in Drosophila and in grasshopper embryos. Proc Natl Acad Sci USA 1982; 79: 2700-2704.

[17] Hedwig B, Knepper M. NEUROLAB, a comprehensive program for the analysis of neurophysiological and behavioural data. J Neurosci Meth 1992; 45: 135-148.

[18] Pfahlert C, Lakes-Harlan R. Responses of insect neurons to neurotrophic factors *in vitro*. Naturwissenschaften 1997; 84: 163-165.

[19] Bicker G, Schäfer S, Ottersen OP, Storm-Mathisen J. Glutamate-like immunoreactivity in identified neuronal populations of insect nervous systems. J Neurosci 1988; 8: 2108-2122.

[20] Watson AHD, Laurent G. GABA-like immunoreactivity in a population of locust intersegmental interneurons and their inputs. J Comp Neurol 1990; 302: 761-767.

[21] Hayashi JH, Hildebrand JG. Insect olfactory neurons *in vitro*: Morphological and physiological characterization of cells from the developing antennal lobes of *Manduca sexta*. J Neurosci 1990; 10: 848-859.

[22] Hayashi JH, Levine RB. Calcium and potassium currents in leg motorneurons during postembryonic development in the hawkmoth *Manduca sexta*. J Exp Biol 1992; 171: 15-42.

[23] Prugh J, Della Croce K, Levine RB. Effects of the steroid hormone, 20-Hydroxyecdysone, on the growth of neurites by identified insect motorneurons *in vitro*. Dev Biol 1992; 154: 331-347.

[24] Fritzsch B. Fast axonal diffusion of 3000 molecular weight dextran amines. J Neurosci Meth 1993; 50: 95-103.

[25] Lakes-Harlan R, Jacobs K, Heinrich R. Identification of auditory interneurons in situ and in vitro by tracer injection into an afferen neuropil of *Locusta migratoria*. Naturwissenschaften 1998; 85: 240-243.

[26] Gray EG. The fine structure of the insect ear. Philos Trans R Soc Lond Biol 1960; 243: 75-94.

[27] Stengl M, Hildebrand JG. Insect olfactory neurons *in vitro*: morphological and immunocytochemical characterization of male-specific antennal receptor cells from developing antennae of male *Manduca sexta*. J Neurosci 1990; 10: 837-847.

[28] Torkkeli PH, French AS. Primary culture of antennal mechanoreceptor neurons of *Manduca sexta*. Cell Tissue Res 1999; 297: 301-309.

[29] Nakagawa A, Iwama A. Dissociated neurons of the pupal blowfly antenna in cell culture. Tissue Cell 1995; 27: 603-612.

[30] Römer H, Marquart V, Hardt M. Organization of a sensory neuropile in the auditory pathway of two groups of Orthoptera. J Comp Neurol 1988; 275: 201-215.

[31] Burrows M, Hoyle G. Neural mechanisms underlying behavior in the locust *Schistocerca gregaria*. III. Topography of limb motorneurons in the metathoracic ganglion. J Neurobiol 1973; 4: 167-186.

[32] Lapied B, Tribut F, Sinakevitch I, Hue B, Beadle DJ. Neurite regeneration of long-term cultured adult insect neurosecretory cells identified as DUM neurons. Tissue Cell 1993; 25: 893-906.

[33] Williams DK, Cohan CS. The role of conditioning factors in the formation of growth cones and neurites from the axon stump after axotomy. Dev Brain Res 1994; 81: 89-104.

[34] Sokoliuk T, Stumpner A, Ronacher B. GABA-like immunoreactivity suggests an inhibitory function of the thoracic low-frequency neuron (TN1) in acridid grasshoppers. Naturwissenschaften 1989; 76: 223-225.

[35] Lees G, Beadle DJ, Botham RP, Kelly JS. Excitable properties of insect neurones in culture: a developmental study. J Ins Physiol 1985; 31: 135-143.

[36] Hoyle G, Burrows M. Neural mechanisms underlying behavior in the locust *Schistocerca gregaria*. I. Physiology of identified motorneurons in the metathoracic ganglion. J Neurobiol 1973; 4: 3-41.

[37] Seshan KR, Provine RR, Levi-Montalcini R. Structural and electrophysiological properties of nymphal and adult insect medial neurosecretory cells: an *in vitro* analysis. Brain Res 1974; 78: 359-376.

[38] Zufall F, Stengl M, Franke C, Hildebrand JG, Hatt H. Ionic currents of cultured olfactory receptor neurons from antennae of male *Manduca sexta.* J Neurosci 1991; 11: 956-965.

[39] Stengl M, Zufall F, Hatt H, Hildebrand JG. Olfactory receptor neurons from antennae of developing male *Manduca sexta* respond to components of the species-specific pheromone *in vitro*. J Neurosci 1992; 12: 2523-2531.

[40] Stengl M. Intracellular-messenger-mediated cation channels in cultured olfactory receptor neurons. J Exp Biol 1993; 178: 125-147.

[41] Hill KG. The physiology of locust auditory receptors. II. Membrane potentials associated with the response of the receptor cell. J Comp Physiol 1983; 152: 483-493.

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