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A muscarinic cholinergic mechanism underlies activation of the central pattern generator for locust flight

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SUMMARY

A central question in behavioural control is how central pattern generators (CPGs) for locomotion are activated. This paper disputes the key role generally accredited to octopamine in activating the CPG for insect flight. In deafferented locusts, fictive flight was initiated by bath application of the muscarinic agonist pilocarpine, the acetylcholine analogue carbachol, and the acetylcholinesterase blocker eserine, but not by nicotine. Furthermore, in addition to octopamine, various other amines including dopamine, tyramine and histamine all induced fictive flight, but not serotonin or the amine-precursor amino acid tyrosine. However, flight initiation was not reversibly blocked by aminergic antagonists, and was still readily elicited by both natural stimulation (wind) and pilocarpine in reserpinized, amine-depleted locusts. By contrast, the muscarinic antagonists atropine and scopolamine reversibly blocked flight initiated by wind, cholinergic agonists, octopamine, and by selective stimulation of a flight-initiating interneurone (TCG). The short delay from TCG stimulation to flight onset suggests that TCG acts directly on the flight CPG, and accordingly that TCG, or its follower cell within the flight generating circuit, is cholinergic. We conclude that acetylcholine acting *via* muscarinic receptors is the key neurotransmitter in the mechanism underlying the natural activation of the locust flight CPG. Amines are not essential for this, but must be considered as potential neuromodulators for facilitating flight release and tuning the motor pattern. We speculate that muscarinic activation coupled to aminergic facilitation may be a general feature of behavioural control in insects for ensuring conditional recruitment of individual motor programs in accordance with momentary adaptive requirements.

Key words: acetylcholine, octopamine, tyramine, invertebrate, identified neurone, behaviour.

INTRODUCTION

Central pattern generators (CPGs), comprising networks of central neurones that can produce the basic motor patterns underlying numerous rhythmic behaviours without sensory timing cues, are frequently studied to gain insights into the mechanisms of motor control. They occur throughout the animal kingdom, and can often be activated by applying neurochemicals (Marder and Bucher, 2001). One of the first identified CPGs underlies flight in locusts (Wilson, 1961; Edwards, 2006). This CPG can be experimentally activated in the isolated nervous system of adult and larval locusts by octopamine (Stevenson and Kutsch, 1987; Stevenson and Kutsch, 1988), which induces plateau potentials in flight interneurones (Ramirez and Pearson, 1991). Octopamine, the invertebrate analogue of noradrenaline (Evans, 1985; Roeder, 1999), is now generally accredited with playing a primary role in flight initiation (for reviews, see Orchard et al., 1993; Libersat and Pflüger, 2004).

However, *Drosophila* null mutants for tyramine-β-hydroxylase (strain: $T\beta H^{nM18}$), which converts tyramine to octopamine, appear capable of normal behaviour, although devoid of octopamine (Monastirioti et al., 1996). They still generate the rhythmic motor pattern for crawling (Fox et al., 2006) and, despite deficits in flight propensity and duration, exhibit normal wing beat amplitudes and frequency, suggesting that octopamine is not essential for flight initiation (Brembs et al., 2007). We speculate that the same may apply to locusts, since none of the identified flight-initiating interneurones appear to be octopaminergic (Stevenson and Spörhase-Eichmann, 1995), and known octopaminergic neurones [e.g. dorsal unpaired median (DUM) cells] do not initiate flight (Libersat and Pflüger, 2004).

So, which neurotransmitters might control flight initiation in insects? $T\beta H^{nM18}$ mutants have tenfold elevated level of tyramine (Monastirioti et al., 1996), which can bind to octopamine receptors (Balfanz et al., 2005) and may thus supplant the action of octopamine (cf. Hardie et al., 2007). However, tyramine is suggested to inhibit flight initiation in *Drosophila* (Brembs et al., 2007). Other transmitter systems are unaffected in $T\beta H^{nM18}$ mutants (Monastirioti et al., 1996). For example dopamine, which is claimed to initiate locomotion and regulate arousal in *Drosophila* (Yellman et al., 1997; Andretic et al., 2005; Kume et al., 2005), also activates flight in moths (Claassen and Kammer, 1986). Furthermore, in contrast to $T\beta H^{nM18}$ mutants, *Drosophila* mutants lacking the receptor for the second messenger inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) are flightless, and evidence suggests this is due to development defects in dopaminergic and/or serotonergic interneurones (Banerjee et al., 2004). In insects $Ins(1,4,5)P_3$ is also involved in neuronal excitation *via* muscarinic acetylcholine receptors (Wenzel et al., 2002) and muscarinic agonists are known to activate various CPGs, including that for locust walking (Ryckebusch and Laurent, 1993). Furthermore, the giant fibre mediating escape in *Drosophila* is one of the few cholinergic interneurones identified so far in insects (Allen and Murphey, 2007).

In the present study we show that cholinergic agonists and several amines in addition to octopamine, all activate the flight CPG in locusts. Amine depletion using reserpine revealed that amines are

not essential. However, since cholinergic antagonists reversibly blocked flight initiation by natural (wind) stimulation, putative neurotransmitters and an identified flight-initiating interneurone (TCG) (cf. Bicker and Pearson, 1983), our data suggest that cholinergic neurones are required for flight initiation in locusts.

MATERIALS AND METHODS

Experimental animals

All experiments were carried out on adult desert locusts (*Schistocerca gregaria* Forskål; gregarious phase) of both sexes taken at least 1 week after the imaginal ecdysis. The specimen were obtained from Blades Biological (Cowden, Kent, UK), maintained under crowded conditions at constant temperature (25°C) and at 45% relative humidity under a light:dark cycle of 12 h:12 h and fed daily on fresh lettuce. The animals were withdrawn just prior to the experiments, which took place in a faraday cage under an incandescent lamp at constant ambient temperature (28°C). The experiments complied with the Principles of Laboratory Animal Care and the German Law on the Protection of Animals (Deutsches Tierschutzgesetz).

Preparation and electrophysiological recording

After amputating the legs and wings at the base, the pronotal shield and the abdomen posterior to the second abdominal segment were cut away. The locusts were opened dorsally by a midline longitudinal incision, the gut pulled out and pinned to one side and the animal fixed to a cork platform, ventral side down. Fat bodies, air sacks and trachea covering the thoracic musculature and the nervous system were carefully removed and the preparation continually superfused with insect saline (140 mmol l^{-1} NaCl, 10 mmol l^{-1} KCl, 7 mmol l^{-1} $CaCl_2$, 8 mmol l^{-1} $NaHCO_3$, 1 mmol l^{-1} $MgCl_2$, 5 mmol l^{-1} *N*-trismethyl-2-aminoethanesulfonic acid, 4 mmol l^{-1} D-trehalose dihydrate, pH 7.4). To eliminate phasic sensory inputs to the flight central pattern generator, the animals were deafferented by severing the connectives to the abdominal ganglia, leaving the anterior connectives to the brain intact, and all nerve branches originating from the meso- and metathoracic ganglia except the four N3A [numbered after Campbell (Campbell, 1961)]. This nerve contains the motor axons of several wing depressor and elevator muscles [after Snodgrass (Snodgrass, 1929): M83, 84, 89, 97, 98, 113, 118, 127, 128], several auxiliary flight muscles, the common inhibitor neurone and several DUM neurones (cf. Siegler and Pousman, 1990). This nerve is not known to innervate sense organs, but the existence of sensory axons cannot be entirely excluded.

Major features of the flight motor pattern were evaluated from extracellular recordings from the same set of flight muscles in all experiments using bipolar stainless steel wire electrodes insulated to the tip (100 μm) and a silver ground wire in the bathing medium: the right hindwing elevator (M113, $E_{h\text{-}r}$) and depressor muscle (M127, $D_{h\text{-}r}$), together with the latter muscle's left side fore- (M97, $D_{f\text{-}l}$) and hindwing homologues (M127, $D_{h\text{-}l}$).

In order to record and stimulate the tritocerebral giant interneurone (TCG), the legs and wings were amputated at the base, the abdomen cut away posterior to the second abdominal segment, the mouthparts removed and the animals mounted ventral side up on a cork platform. The tritocerebral commissure was then exposed after carefully withdrawing the gut. The TCG interneurone was recorded and stimulated extracellularly using bipolar steel hook electrodes formed from electrolytically sharpened fine tungsten steel pins placed under the posterior branch of the tritocerebral commissure, which contains only the axons of the tritocerebral giant (TCG) and dwarf (TCD) interneurones (Bacon and Tyrer, 1978). Stimulation was achieved using an isolated stimulator (Grass SD9; Grass Medical Instruments, Quincy, MA, USA) with 0.1 ms pulses set slightly above the threshold voltage for recruitment of the TCG, as checked by an extracellular recording from the ipsilateral pro-mesothoracic connective. In these experiments, flight motor activity was monitored by bipolar electrodes (tungsten steel pins, 200 μm) inserted through the cuticle on the ventral side in the first basalar depressor muscles (M127) of both hindwings.

All recordings were amplified by differential amplifiers (University of Leipzig), digitalized (PowerLab 8/30, ADInstruments Pty Ltd., Bella Vista, NSW, Australia: sampling frequency 10 kHz, ADC resolution 16 bit) and stored using standard software (Chart and Scope, ADInstruments) running on a Power Macintosh computer (Apple Computers, Cupertino, CA, USA).

Flight initiation and pharmacological treatments

Natural initiation of flight motor activity was achieved by delivering wind (approx. 6 m s^{-1}) to the head hairs from a commercial hairdryer (LLD 800; AKA Electric, Berlin, Germany). This served as a reference for motor activity initiated by pharmacological agents.

The flight-initiating capacity of neurochemicals was tested by exchanging the saline perfusion for freshly prepared test solutions using a manually operated two-way valve. Unless otherwise stated all drugs were obtained from Sigma-Aldrich GmbH (Steinheim, Germany). DL-octopamine hydrochloride, tyramine hydrochloride, dopamine hydrochloride, histamine dihydrochloride, epinephrine bitartrate (adrenaline), norepinephrine hydrochloride (noradrenaline), serotonin hydrochloride, acetylcholine chloride, carbamylcholine chloride (carbachol), pilocarpine hydrochloride, nicotine hemisulphate, physostigmine sulphate (eserine; Research Biochemicals Inc., Natick, MA, USA) were dissolved in insect saline at the empirically determined lowest effective concentration (see Results). Tyrosine hydrochloride was first dissolved in 1 mol l^{-1} HCl and then diluted with insect saline and neutralized with 1 mol l^{-1} NaOH to pH 7–8.

Neurotransmitter antagonists (atropine sulphate, scopolamine hydrochloride, tubocurarine chloride hydrate, epinastine hydrochloride; Boehringer Ingelheim, Germany; phentolamine hydrochloride, propranolol hydrochloride; Research Biochemicals Inc.) were perfused for at least 20 min prior to testing their ability to block the flight-initiating action of wind, neurotransmitter agonists (applied together with the antagonist) and TCG stimulation.

Reserpine, a non-specific amine depleter, was dissolved in dimethylsulphoxide (DMSO) to give a final concentration of 50 mg ml^{-1}. Animals received two applications of 5 μl of this solution, injected in the thoracic cavity with a microsyringe (Hamilton, Bonaduz, Switzerland), 3 and 1 day prior to the experiment, giving 500 μg reserpine per locust.

Amine immunocytochemistry

Octopamine depletion by reserpine was checked by immunocytochemistry using a specific rabbit polyclonal octopamine antiserum on paraffin sections (10 μm) by the standard avidin–biotin technique using diaminobenzidine as chromogen as described in detail elsewhere (Stevenson et al., 1992). Sections were viewed with a compound microscope (Leitz DMR; Leica, Wetzlar, Germany) using phase interference contrast (Nomarski) optics. Images were obtained with a mounted CCD camera (SensiCam; PCO Computer Optics, Kelheim, Germany) using automatic exposure and colour/brightness compensation. Images were scaled, trimmed and converted to 300 d.p.i. 8-bit using standard software (Canvas X; ACD Systems, Saanichton, BC, Canada) running on a Power

Macintosh computer. Beyond this, no further image processing was undertaken.

Data analysis

Flight muscle activity was evaluated from 20 consecutive fictive flight cycles of five different preparations for each test group. Muscle activation times were measured manually using the cursor function of standard software (Chart and Scope) and the means and standard deviations of the following parameters calculated (Excel, Microsoft Corporation, Redmond, WA, USA): flight cycle (DD) as the interval between the first firing of a burst and the first firing of the next burst of the right hindwing depressor (M127) and from this rhythm frequency (F); left–right wing latency (LR) and hind–forewing latency (HF) from the first potential of bursts of this depressor muscle in relationship to the first potential of bursts of its homologues; elevator depressor (ED) and depressor elevator (DE) latencies from the first potentials of bursts of the right hindwing depressor and its functional antagonist (M113); phase of the elevator in the depressor cycle from DE/DD. Student's two-tailed *t*-test (unpaired and paired as appropriate) was applied to test for statistical significance of differences between means using standard software (GBStat 6.5, Dynamic Microsystems, Silver Spring, MD, USA). Charts were finally arranged using Canvas X.

RESULTS

Wind-induced fictive flight

For comparative purposes, Fig. 1A shows an example of flight motor activity ('fictive flight') elicited by the natural releasing stimulus (wind) as recorded from example wing elevator and depressor muscles of a deafferented locust preparation. Confirming previous studies (e.g. Stevenson and Kutsch, 1987), a detailed analysis of this pattern (Fig. 2, Table 1) revealed the following defining features. (1) All motor units were activated rhythmically at the same overall frequency of about half the wing-beat frequency of intact locusts (8.6±1.5 Hz; mean ± s.d.) (Fig. 2A), whereby wing elevator units tend to be activated more often per cycle after deafferentation. (2) Elevator ($E_{h\text{-}r}$) and depressor ($D_{h\text{-}r}$) muscles were activated alternately, whereby the depressor–elevator latency (DE latency: 71±15 ms; mean ± s.d.) is longer than the elevator–depressor latency (ED latency: 51±13 ms; Fig. 2B), so that the phase of the elevator in the depressor cycle (DE/DD) is greater than 0.5 (0.58±0.06; Fig. 2C). (3) Hindwing depressor muscles ($D_{h\text{-}l}$) were activated in advance of their forewing homologues ($D_{v\text{-}l}$; HF latency: 19±6 ms; Fig. 2D). (4) Homologous muscles of the left and right hindwing ($D_{h\text{-}r}$, $D_{h\text{-}l}$) were activated synchronously (LR latency: –0.1±3.6 ms; Fig. 2E).

Cholinergic-induced fictive flight

Superfusing the thoracic ganglia with the muscarinic agonist pilocarpine was found to elicit fictive flight within 5–15 s after its application and most effectively at a concentration of 5 mmol l^{-1} (53 of 56 preparations). This response lasted several minutes and typically comprised fictive flight sequences alternating with silent periods in all muscles (Fig. 1B, upper trace). The first flight activity phase was always the longest and lasted up to 2 min. After this there was no consistent pattern in the durations of the active and silent phases, which both varied throughout a sequence and between preparations from seconds to minutes. Details of the motor pattern, however, were consistent throughout the whole periods of activity (Fig. 1B, lower traces) and corresponded in all defining features to wind-induced fictive flight. Compared to wind, the frequency of pilocarpine-induced flight was somewhat higher (11.5±1.9 Hz; Fig. 2A, Table 1) and, as in intact locusts (cf. Weis-Fogh, 1956) individual muscles were usually only activated once per cycle. Hindwing depressor units led the forewing homologous units, though with a shorter time lag (5±4 ms), and the left and right side homologous units were activated in near synchrony (mean latency 0.9±3.2 ms; Fig. 2D,E, Table 1). The depressor to elevator latency was slightly longer than the reverse period and the phase of the elevator in the depressor cycle (0.52±0.04) was not statistically different from that for wind-induced flight (Fig. 2B,C, Table 1). Pilocarpine (5 mmol l^{-1}) also induced flight motor activity in isolated pterothoracic ganglia preparations, and there was no obvious difference to the fictive flight pattern evaluated for deafferented locusts (*N*=3, data not shown).

The naturally occurring neurotransmitter acetylcholine evoked minute long continuous sequences of flight muscle activity, although this was typically uncoordinated at the minimum effective doses (100 mmol l^{-1}; *N*=20; Fig. 1C) and did not alter with higher concentrations. There was mostly no clear indication of rhythmic bursting in the elevator and depressors motor units, which were often activated simultaneously. In three preparations, however, short periods (up to 15 s) of fictive flight were evident (not evaluated in detail) within a continuous sequence. The example in Fig. 1C shows a short section of a transitory sequence between non-rhythmicity and fictive flight, during which depressor motor units become synchronized, though not yet in strict alternation with elevator motor units.

Eserine had been previously reported to induce hyperactivity in insects (Roeder, 1939; Kutsch and Murdock, 1973), and so this inhibitor of acetylcholinesterase was tested, using 1 mmol l^{-1}, which induced short (20–30 s) bouts of fictive flight in all three preparations tested (Fig. 1D; pattern not evaluated in detail). This suggests that naturally released acetylcholine has the potential to induce flight, and that the relative ineffectiveness of acetylcholine superfusion may be due to the abundance of acetylcholinesterase in insect nervous tissues (Treherne and Smith, 1965). We thus investigated the effect of carbachol, a nonhydrolysable analogue of acetylcholine. This compound readily (10 of 18 preparations) induced long-lasting activity in flight muscles, even at the comparatively low concentration of 5 mmol l^{-1} (Fig. 1E). At its onset, this motor activity generally appeared somewhat irregular, but clear coordination of the flight muscles was established within several seconds and this remained stable and continued for 20 min or longer. The frequency of carbachol-induced fictive flight was extraordinarily high for deafferented locusts [maximum 21 Hz; 18.1±3.5 Hz (mean ± s.d.); Fig. 2A, Table 1] and within the range of the wing beat frequency of intact tethered and freely flying locusts [20–25 Hz (Kutsch and Stevenson, 1981)]. All major features of the normal flight motor pattern were evident (Fig. 2, Table 1).

In contrast to the above, the agonist nicotine only evoked uncoordinated motor activity (*N*=6; Fig. 1F). At its minimum effective dosage (1 mmol l^{-1}), the response occurred immediately and was characterised by high frequency discharges of all recorded flight muscles lasting 10–15 s, with no indication of temporal coupling between different units. This was followed by a long period of inactivity, during which fictive flight could not be induced by wind or pilocarpine.

Flight induction and cholinergic antagonists

Flight initiation was reliably and reversibly blocked by muscarinic antagonists (Fig. 3). In preparations that readily produce flight in response to wind stimulation (e.g. Fig. 3Ai), flight induction by wind was completely inhibited in the presence of the muscarinic

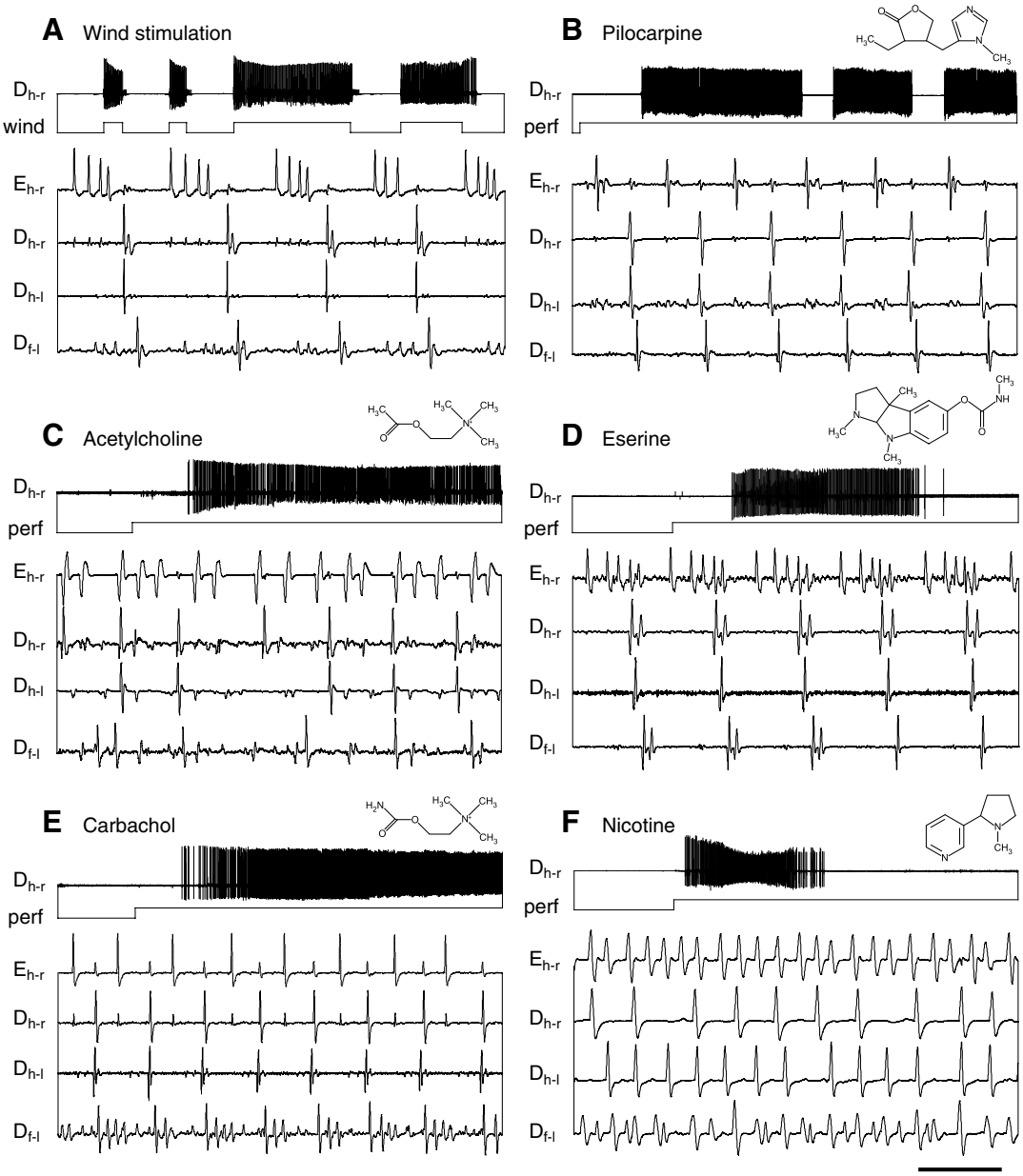

Fig. 1. Electromyograms of flight motor activity induced by (A) wind stimulation (wind, ~6 m s^{-1}) compared to motor activity evoked by bath applied cholinergic agonists (perf, B–F) in deafferented locust preparations. The top traces of each panel show continual sequences as recorded from the right hindwing depressor muscle ($D_{h\text{-}r}$) and the lower traces show details of the pattern as recorded from the right hindwing elevator ($E_{h\text{-}r}$) and depressor ($D_{h\text{-}r}$) and the depressor left fore- ($D_{f\text{-}l}$) and hindwing homologous ($D_{h\text{-}l}$) muscles. (B) The muscarinic agonist pilocarpine (5 mmol l^{-1}) initiates flight motor activity interrupted by pauses. (C) Acetylcholine (100 mmol l^{-1}) induces continuous rhythmic motor activity with occasional interspersed sequences that resemble flight. (D) Eserine (1 mmol l^{-1}) induces a short flight sequence. (E) The cholinergic agonist carbachol (5 mmol l^{-1}) induces flight motor activity at exceptionally high frequency. (F) Nicotine (1 mmol l^{-1}) induces a short burst of uncoordinated motor activity only. Scale bar, 10 s upper traces, 100 ms lower traces.

cholinergic antagonist atropine (10 mmol l^{-1}, N=12; Fig. 3Aii). Regardless of intensity and duration of the wind stimulus, only 2–5 spikes in elevator motor units, if anything, were monitored (Fig. 3Aii). After prolonged washing with insect saline (20 min), the response to wind was completely restored (Fig. 3Aiii). Similarly, pilocarpine (5 mmol l^{-1}) also failed to initiate fictive flight in the presence of atropine (10 mmol l^{-1}, N=13; Fig. 3B). The depicted example shows a sequence with the highest degree of motor activity evoked by pilocarpine under atropine. In most cases we observed no motor response. Corresponding results were obtained for the muscarinic antagonist scopolamine (10 mmol l^{-1}, N=6; Fig. 3C). We were unable to achieve a selective, reversible blockade of motor activity with the nicotinic antagonist tubocurarine. Whereas a 10 mmol l^{-1} solution failed to block the uncoordinated activity typically evoked by nicotine (cf. Fig. 1F), higher concentrations abolished all motor activity irreversibly (N=5, not shown).

Neuronal flight induction and muscarinic antagonists

Contrary to other flight-initiating interneurones (cf. Pearson et al., 1985), the flight-initiating tritocerebral giant interneurone [TCG (cf. Bicker and Pearson, 1983)] can be accessed by extracellular electrodes (Fig. 4A). Its large diameter axon (20 µm) descends from the brain

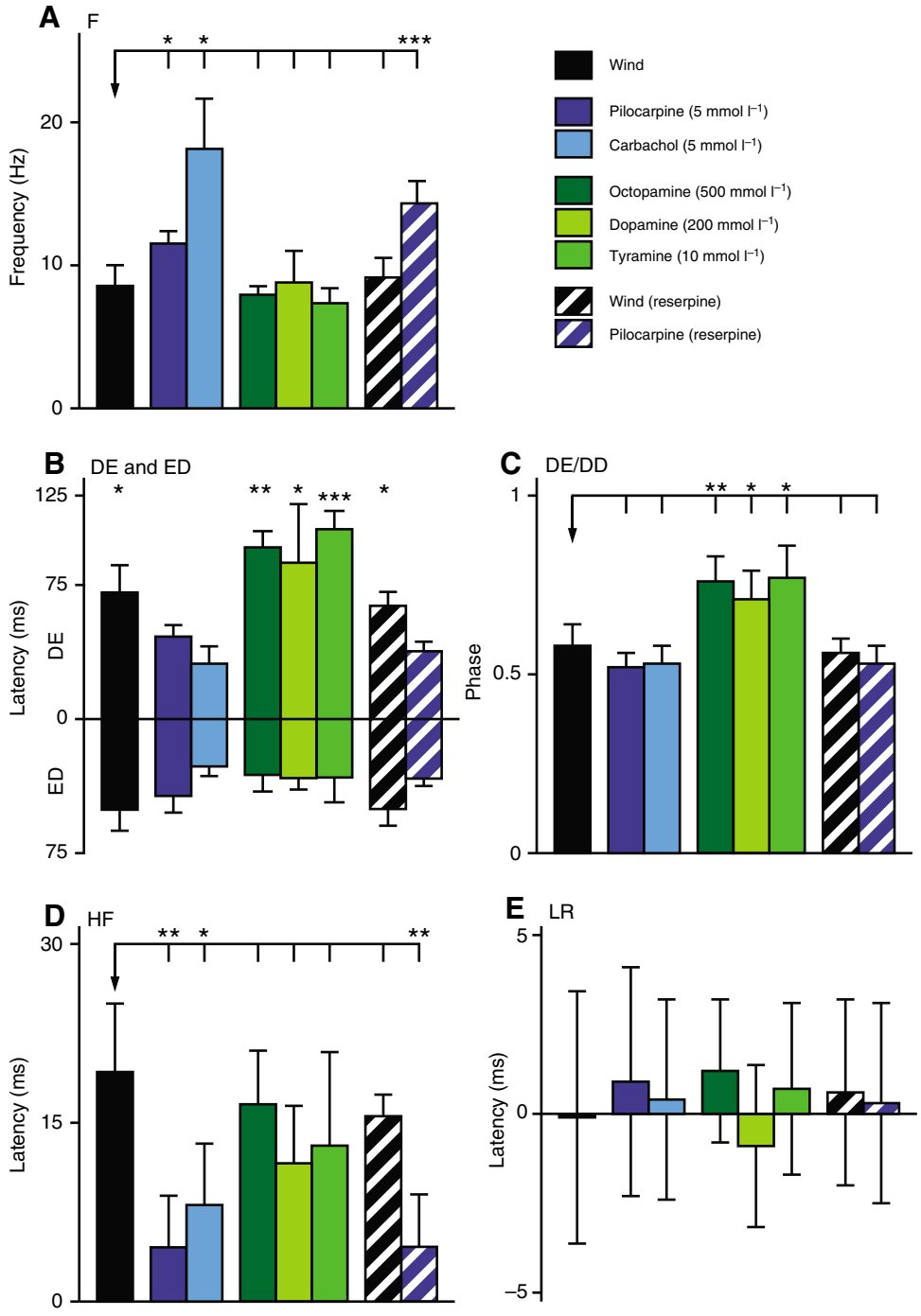

Fig. 2. Comparison of key features of the flight motor pattern in deafferented locust preparations released by various treatments: (from left to right) wind stimulation, pilocarpine, carbachol, octopamine, dopamine, tyramine and finally by wind and pilocarpine after amine depletion. Values are means + s.d., from 100 cycles, 20 from each of five animals for each condition. (A) Rhythm frequency. Note the elevated frequency of the cholinergic-induced patterns. (B) Depressor elevator (DE) and elevator depressor (ED) latencies. The DE latency is longer than the ED latency. (C) Phase. The phase of the elevator in the depressor cycle is greater for flight released by the amines. (D) Hind–forewing (HF) latency. The forewing depressor muscles lag several milliseconds behind the homologous hindwing muscles in all cases. (E) Left–right wing (LR) latency. The homologous depressor muscles of the two body sides are activated in near synchrony for all treatments. Asterisks in A, C and D indicate significant differences from the wind-induced flight motor pattern (unpaired two-tailed *t*-test); asterisks in B indicate significant differences between the DE and ED latencies (paired two-tailed *t*-test). **P*<0.05; ***P*<0.01; ****P*<0.001.

to the thoracic ganglia *via* the posterior arc of the tritocerebral commissure (tcc; Fig. 4A), which contains the axon of only one additional neurone, the smaller tritocerebral dwarf [TCD, 5 µm (Bacon and Tyrer, 1978; Tyrer et al., 1988)]. Spikes of the TCG are conducted at about the same velocity as other locust giant interneurones [3.74±0.19 m s^{-1}, *N*=5, 28°C (compare with Boyan and Ball, 1990)] and are readily distinguished by the prominent response to wind stimulation and their greater size. Furthermore, TCG can be selectively recruited by stimulating the tritocerebral commissure at the threshold for evoking its spike in the thoracic connective (3–8 V, 0.1 ms), which is unlikely to recruit the smaller TCD.

Repetitive (but not single) extracellular stimulation (20, 0.1 ms pulses, 200 Hz) of the TCG induced fictive flight in 14 of 38 preparations, and then even several times in succession without fail (Fig. 4Bi). The recorded activity always started in the elevator muscles of both sides synchronously and began as soon as 23.3 ms (minimum, mean 34.4±8.5 ms; five trials each for five animals) after the first stimulus pulse. This initial elevator burst was followed by a depressor burst and sustained alternating activity of both muscles as in fictive flight (Fig. 4Ci) that typically lasting several seconds and on one occasion even 80 s.

In the presence of atropine (10 mmol l^{-1}, *N*=5) perfused locally over the thoracic ganglia, repetitive electrical stimulation of TCG failed to evoke fictive flight, or indeed any motor response of the flight muscles (Fig. 4Bii,Cii). This blockade was reversible in that the capacity of TCG stimulation to evoke fictive flight was

Table 1. Values of key features of the flight motor pattern in deafferented locust preparations released by various treatments

	F (Hz) (t/P values)*	DE (ms)	(t/P values)†	ED (ms)	DE/DD phase (t/P values)*	HF (ms) (t/P values)*	LR (ms) (t/P values)*
Wind	8.6±1.5	71±15	(3.40/0.03)	51±13	0.58±0.06	19±6	–0.1±3.6
Pilocarpine	11.5±1.9 (2.80/0.02)	46±6	(0.97/0.39)	43±11	0.52±0.04 (1.93/0.09)	5±4 (4.57/0.001)	0.9±3.2 (0.49/0.67)
Carbachol	18.1±3.5 (5.64/0.01)	31±9	(1.51/0.20)	26±5	0.53±0.05 (1.51/0.21)	8±5 (3.23/0.02)	0.4±2.8 (0.26/0.81)
Octopamine	7.9±0.6 (0.88/0.51)	96±9	(8.52/0.001)	31±10	0.76±0.07 (4.21/0.003)	17±4 (0.83/0.44)	1.2±2.0 (0.71/0.55)
Dopamine	8.8±2.2 (0.21/0.85)	87±33	(3.54/0.02)	33±7	0.71±0.08 (2.93/0.03)	12±5 (2.28/0.07)	–0.9±2.4 (0.42/0.69)
Tyramine	7.3±1.0 (1.52/0.22)	106±10	(9.92/0.0006)	33±15	0.77±0.09 (3.84/0.02)	13±8 (1.42/0.25)	0.7±2.4 (0.41/0.72)
Wind after reserpine	9.2±1.4 (0.66/0.57)	63±8	(3.65/0.02)	50±10	0.56±0.04 (0.83/0.43)	16±2 (1.38/0.23)	0.6±2.6 (0.39/0.74)
Pilocarpine after reserpine	14.3±1.6 (6.05/0.0006)	38±5	(1.43/0.23)	33±5	0.53±0.05 (1.54/0.20)	5±4 (4.53/0.002)	0.3±2.8 (0.20/0.85)

Values are means ± s.d. from 100 cycles, 20 from each of five animals for each condition. t/P values (in parentheses) are from Student's two-tailed t-test: *unpaired, tested against wind data; †paired, tested between DE and ED latencies.

F, flight-rhythm frequency; DE, depressor to elevator latency; ED, elevator to depressor latency; DE/DD phase, elevator phase in depressor cycle; HF, hind to forewing latency; LR, left to right wing latency.

completely restored after flushing the thoracic ganglia for 30 min with insect saline in all five preparations (Fig. 4Biii,Ciii).

Amine-induced fictive flight

We show here that in addition to octopamine (cf. Sombati and Hoyle, 1984; Stevenson and Kutsch, 1987), a variety of other amines also have the capacity to induce fictive flight in deafferented locusts (Figs 2 and 5, Table 1).

Of the amines tested, octopamine in fact appeared to be the least effective, in that fictive flight was first reliably induced by a 500 mmol l^{-1} solution (34 of 37 preparations; Fig. 5A), only occasionally by 100 mmol l^{-1} and then only after prolonged superfusion. These flight sequences were typically induced within several seconds after application and lasted some 1–4 min. Corresponding results were found for the trace amines noradrenaline and adrenaline that induced flight at a concentration of 500 mmol l^{-1} (not shown). The structurally related catecholamine dopamine appeared to be more effective in that it induced fictive flight at a lower concentration (200 mmol l^{-1}, eight of nine preparations; Fig. 5B), although here the fictive flight sequences were typically interrupted by short silent periods. Histamine, reliably elicited 1–3 min long bouts of fictive flight at an even lower concentration (minimum 10 mmol l^{-1}, seven of 14 preparations, not shown), but only after several minutes of continuous superfusion. Tyramine, the metabolic precursor of octopamine, appeared to be the most potent flight-inducing amine in that even a 10 mmol l^{-1} solution reliably evoked 2–5 min long sequences of continuous fictive flight within seconds of superfusion (eight of nine preparations; Fig. 5C). A detailed analysis revealed that the flight motor pattern evoked by each of the above amines corresponded in all major respects to the wind-induced flight motor pattern. However, values for the phase of the elevator in the depressor cycle were in all cases significantly greater than observed for wind and cholinergic agonists (Fig. 2, Table 1).

In contrast to the above, we were unable to induce fictive flight with either the indolamine serotonin (10–500 mmol l^{-1}, N=10; Fig. 5D) or the amine-precursor amino acid tyrosine (saturated solution: 10 mmol l^{-1}, N=4; Fig. 5E), which at best evoked only brief periods of uncoordinated activity in some flight muscles.

Flight induction and aminergic antagonists

In contrast to the effect of muscarinic antagonists, we were unable to selectively and reversibly block flight initiation with amine receptor antagonists (data not shown). Briefly, wind still induced flight in the presence of the α-adrenergic antagonist phentolamine (10 mmol l^{-1}, N=4), which also blocks octopamine receptors (Evans, 1981; Roeder, 1995). Phentolamine did inhibit flight induction by octopamine (three of four preparations), but so did the β-adrenergic antagonist propranolol (10 mmol l^{-1}, N=3), which has a low affinity for octopamine receptors (Evans, 1981; Roeder, 1995). The specific octopamine receptor antagonist epinastine (cf. Roeder et al., 1998) failed to inhibit flight induction by both wind and octopamine (N=10) at a dosage known elsewhere to inhibit octopamine [1 mmol l^{-1} (cf. Roeder et al., 1998; Stevenson et al., 2005)]. Although higher dosages of epinastine blocked flight induction (10 mmol l^{-1}, N=11), the effect was irreversible and thus probably non-specific. However, the muscarinic antagonists atropine (10 mmol l^{-1}, N=6; Fig. 5F) and scopolamine (10 mmol l^{-1}, N=3; not shown) both readily and reversibly blocked flight initiation by 500 mmol l^{-1} octopamine.

Flight induction and amine depletion

As an alternative to aminergic antagonists, we investigated the effect of reserpine treatment, which non-specifically depletes stores of biogenic amines in insects [locust (Robertson, 1976); cockroach (Sloley and Owen, 1982); cricket (Stevenson et al., 2000)]. The effectiveness of reserpine treatment was verified by octopamine immunocytochemistry. In control animals (DMSO-injected, 2×5 µl, N=5), the somata and primary neurites of the well-known octopaminergic dorsal unpaired median (DUM) neurones of thoracic ganglia all expressed strong octopamine-like immunoreactivity (Fig. 6A, arrow) as did numerous fine dendritic processes, for

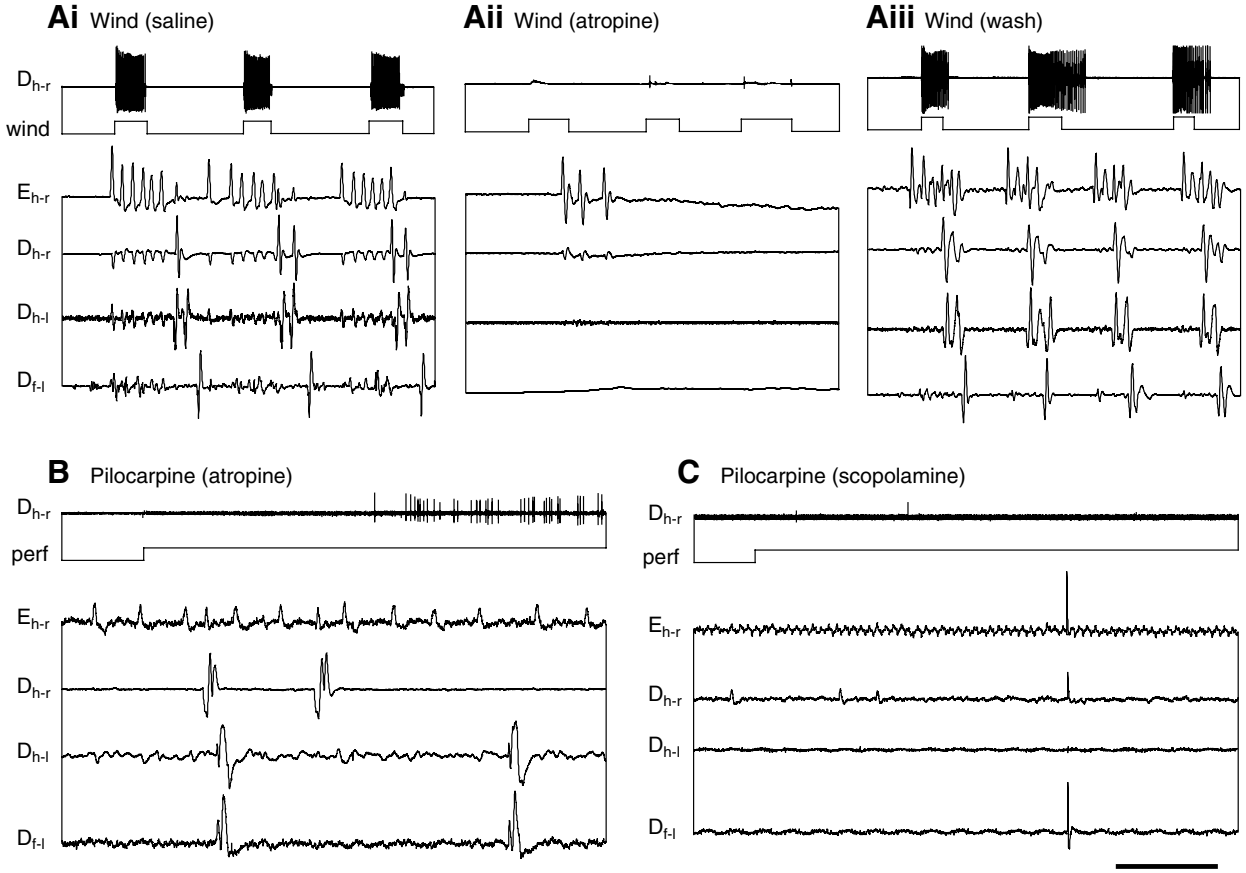

Fig. 3. Reversible muscarinic blockade of flight initiation. (Ai,Aii,Aiii) Responses to wind before, during and after (wash) perfusion with atropine (10 mmol l^{-1}) in the same deafferented preparation. (B) Atropine (10 mmol l^{-1}) and (C) scopolamine (10 mmol l^{-1}) both block flight initiation by pilocarpine (5 mmol l^{-1}). Top traces show condensed, and lower traces expanded excerpts of electromyograms as in Fig. 1. Scale bar: 10 s upper traces, 100 ms lower traces.

example in the dorsal (flight) neuropil (Fig. 6A, insert). By contrast, in animals pre-treated with reserpine (2×250 µg in 5 µl^{-1} DMSO, *N*=5), the DUM cells showed no immunoreactivity (Fig. 6B, arrow) and all neuropils were void of octopamine-immunoreactive stain (Fig. 6B, insert). Corresponding results were found for the cerebral ganglia (not shown).

Approximately a quarter of the locusts pre-treated with reserpine died, while the remainder appeared lethargic and apathetic, but still exhibit various behaviours such as feeding, walking and grooming. Furthermore, fictive flight was readily evoked by wind in nine of ten deafferented preparations of these animals (Fig. 6C), and the exhibited flight motor pattern was in no way different to that evoked by wind in untreated animals (Fig. 2, Table 1). Finally, pilocarpine (5 mmol l^{-1}) was equally effective at initiating fictive flight in reserpinized locusts (eight of nine preparations; Fig. 6D), whereby the flight motor pattern was not different to that in untreated animals (Fig. 2, Table 1), indicating that the action of this muscarinic agonist does not depend on amines.

DISCUSSION

Cholinergic initiation of flight

We claim that the primary mechanism underlying flight initiation in the locust is cholinergic. Firstly, cholinergic agonists applied locally to the deafferented thoracic nervous system induced fictive flight that corresponded in all major respects to the motor pattern induced by wind in deafferented (Figs 1 and 2) and intact locusts (cf. Stevenson and Kutsch, 1987). Secondly, cholinergic antagonists reversibly blocked the release of fictive flight by natural (wind) stimulation (Fig. 3A), cholinergic and aminergic agonists (Fig. 3B,C and Fig. 5F), and an identified flight-initiating interneurone (TCG; Fig. 4).

In addition to flight, cholinergic agonists evoke numerous other motor patterns in insects (Gorczyca et al., 1991; Ryckebusch and Laurent, 1993; Büschges et al., 1995; Ocker et al., 1995; Heinrich et al., 1997) and crustaceans (Marder and Eisen, 1984; Chrachri and Clarac, 1987; Braun and Mulloney, 1993). In these studies, the drug concentration required to induce fictive behaviour, the time till its onset and its duration correspond to our observations for flight (concentration: 5 mmol l^{-1}; onset: 5–10 s; duration: minutes). The effective concentration in nervous tissue is probably less, since the insect blood–brain barrier hinders the passage of solutes (Schofield et al., 1984) (for a review, see Carlson et al., 2000).

Muscarinic actions

As is likely for insect walking (Ryckebusch and Laurent, 1993), cholinergic-released flight probably involves activation of muscarinic rather than nicotinic receptors. Flight initiated both by pilocarpine, a selective muscarinic receptor agonist, and by wind, was reversibly blocked by the muscarinic receptor antagonists atropine and scopolamine (10 mmol l^{-1}; Fig. 3). Two, G-protein-coupled, muscarinic receptor subtypes are known in insects (Breer and Sattelle, 1987; Knipper and Breer, 1988; Trimmer, 1995),

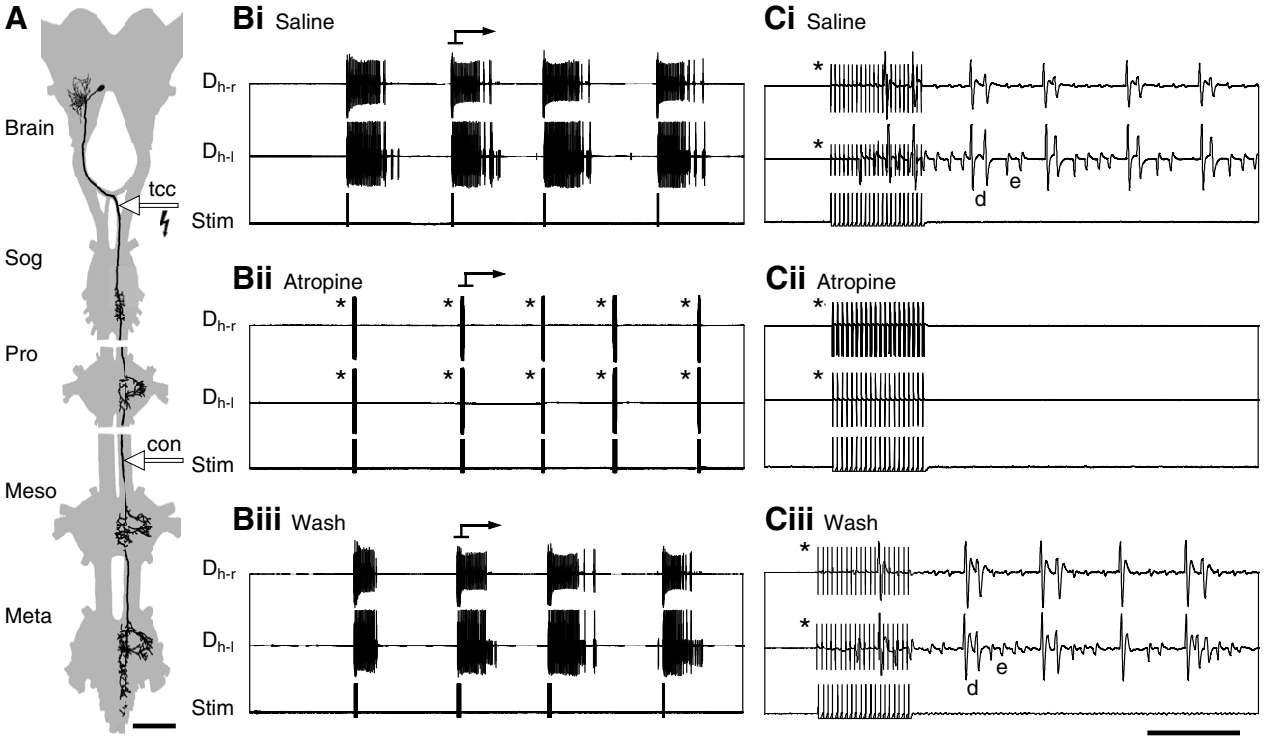

Fig. 4. Flight initiated by stimulating the tritocerebral commissure giant interneurone (TCG) and its reversible blockade by atropine. (A) Pictogram of the TCG interneurone [after Bacon and Tyrer (Bacon and Tyrer, 1978)] showing the site of electrical stimulation (tcc, posterior tritocerebral commissure) and recording (con, connective; Sog, Pro, Meso, Meta, suboesophageal and the three thoracic ganglia, respectively). (Bi) TCG stimulation (stim; 20, 0.1 ms, 5 V pulses, 200 Hz) evokes bouts of flight muscle activity as shown by electromyograms of right ($D_{h\text{-}r}$) and left ($D_{h\text{-}l}$) hindwing depressor muscles. (Ci) Expanded record of the sequence marked in Ai (arrow). Note the cross talk of an elevator muscle (e) revealing rhythmic alternation with depressor muscle activity (d). Asterisks mark stimulus artefacts. (Bii,Cii) Same recordings and animal showing that flight initiation by TCG stimulation is fully blocked during atropine (10 mmol l^{-1}) superfusion and completely restored after washing with saline (Biii,Ciii). Scale bars, A, 1 mm; B, 10 s; C, 100 ms.

postsynaptic receptors that regulate neurone excitability and presynaptic autoreceptors that inhibit acetylcholine release (Trimmer and Weeks, 1993; Leitch and Pitman, 1995), but they cannot yet be distinguished pharmacologically (Honda et al., 2007). In grasshoppers, muscarinic excitation involves both the AC–cAMP–PKA (adenylate cyclase–cyclic adenosine monophosphate–protein kinase A) and PLC–Ins(1,4,5)P_3–DAG (phospholipid C–inositol 1,4,5-trisphosphate–diacylglycerine) pathways, whereby the latter may control stridulation (Wenzel et al., 2002). Interestingly, *Drosophila* Ins(1,4,5)P_3-receptor mutants are flightless, although this is claimed to be due to developmental defects in dopaminergic or serotonergic interneurones (Banerjee et al., 2004).

Nicotinic actions

Nicotine evoked only a short burst of erratic motor activity (Fig. 1F) as also reported for *Drosophila*, where pilocarpine activates feeding (Gorczyca et al., 1991). Interestingly though, whereas fictive flight induced by pilocarpine was interrupted by irregular silent periods, the non-selective agonist carbachol (5 mmol l^{-1}) induced continuous sequences with an exceptionally high rhythm frequency (mean 18 Hz; Fig. 1E) approaching the wing-beat frequency of intact locusts [20–23 Hz (Kutsch and Stevenson, 1981)]. Similarly, carbachol evokes a high frequency swimmeret rhythm in crayfish by combining muscarinic and nicotinic actions (Braun and Mulloney, 1993). In locusts, flight frequency is increased by phasic inputs from wing-hinge stretch receptors (Möhl, 1985), which like other insect mechanoreceptors are cholinergic (for a review, see Homberg, 1994) and operate *via* nicotinic postsynaptic receptors (Leitch and Pitman, 1995; Gauglitz and Pflüger, 2001). Considering this, we conclude firstly that nicotinic mechanisms are probably essential for high-frequency uninterrupted output of the flight CPG, though not for its activation. Secondly, muscarinic agonists are unlikely to evoke flight by mimicking the action of flight-initiating afferents [e.g. wind hairs (Weis-Fogh, 1949); chordotonal organs (Stevenson, 1997)].

Acetylcholine and the TCG interneurone

The failure of acetylcholine to initiate stable fictive flight (100 mmol l^{-1}; Fig. 1C) may result from nicotinic effects masking muscarinic effects (see also Benson, 1992; Heinrich et al., 1997; Heinrich et al., 2001; Wenzel et al., 2002) owing to the greater abundance of nicotinic receptors (Breer, 1981), and the action of acetylcholinesterase (Treherne and Smith, 1965), which would not affect the non-hydrolysable acetylcholine analogue carbachol. Supporting this, the cholinesterase inhibitor eserine (1 mmol l^{-1}), which leads to accumulation of spontaneously released acetylcholine in the synaptic cleft, readily induced flight. That endogenous acetylcholine releases flight is verified by our finding that the action of the flight-initiating interneurone TCG (cf. Bicker and Pearson, 1983) was reversibly blocked by atropine (Fig. 4).

The delay from the first TCG stimulus to the first spike in the initial elevator muscle cycle was only 23 ms (minimum). Deducting the conduction time of TCG to the thoracic ganglia [5 ms (Bacon

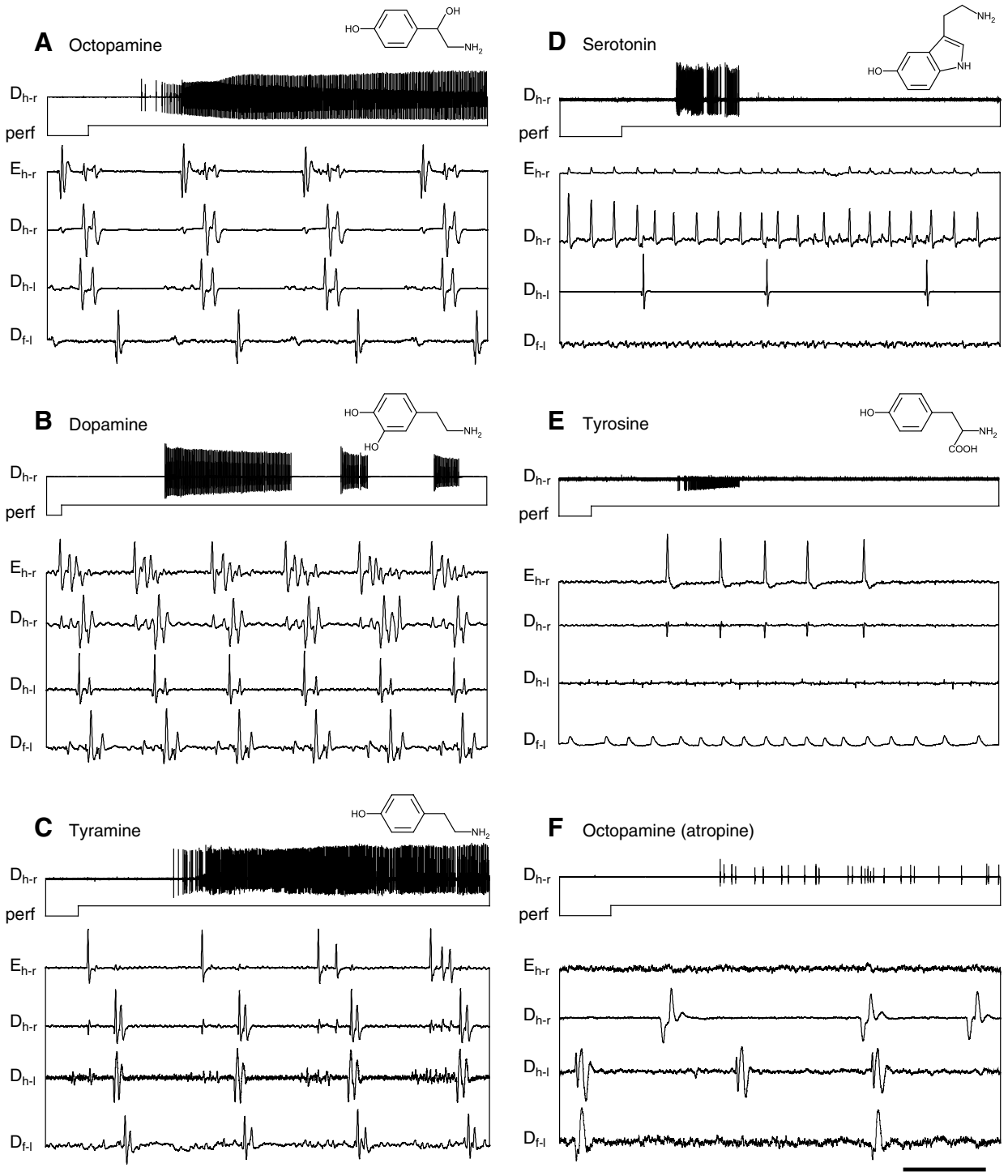

Fig. 5. Amine-induced flight motor activity. Top traces: initial response to bath application; lower traces: expanded excerpts of electromyograms, as in Fig. 1. (A) Octopamine (500 mmol l^{-1}), (B) dopamine (200 mmol l^{-1}) and (C) tyramine (10 mmol l^{-1}) all induce fictive flight, but not (D) serotonin (10 mmol l^{-1}) or (E) the precursor amino acid tyrosine (10 mmol l^{-1}). (F) Atropine (10 mmol l^{-1}) blocks flight initiation by octopamine. Scale bar, 10 s upper traces, 100 ms lower traces.

and Tyrer, 1979)] and from flight motoneurones to muscles [3 ms (Stevenson, 1997)] leaves only 15 ms to activate the flight centre, and even less considering that more than one stimulus is needed. This delay is well within the time required by flight interneurones to drive wing elevator motoneurones [e.g. 13 ms for *514* (Robertson and Pearson, 1983)]. Thus, although only weak synaptic connections with flight motoneurones are presently known (Bacon and Tyrer, 1979; Tyrer, 1980), our data suggest that TCG acts directly on the flight CPG. Accordingly either TCG or its direct follower cell within the flight-generating circuit must be cholinergic. Of the few cholinergic interneurones identified so far in insects (Casagrand and Ritzmann, 1992a; Leitinger and Simmons, 2000; Rind and Leitinger,

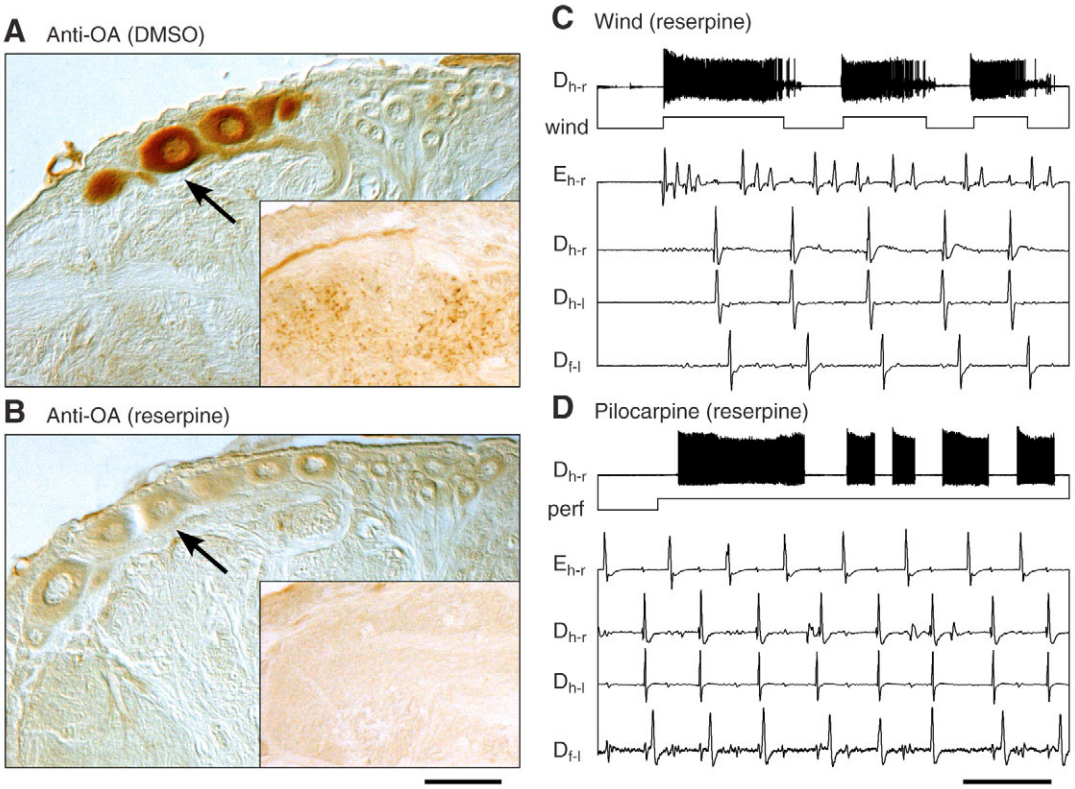

Fig. 6. Flight initiation and amine depletion. (A,B) Photomicrographs of sagittal sections of a thoracic ganglion (anterior left, dorsal top) processed for octopamine immunocytochemistry. (A) In DMSO-treated locusts the well-known octopaminergic DUM neurones, for example, are strongly labelled (arrow, Nomarski optics) and immunoreactive varicosities are visible in the dorsal neuropil (insert, normal light microscopy). (B) Corresponding sections from a reserpine pre-treated animal verifies effective depletion of octopamine from the nervous system (arrow: unlabelled DUM neurone; insert: same neuropil region as in A). (C) Wind- and (D) pilocarpine (5 mmol l^{-1}, bath applied)-induced flight motor activity in deafferented locust preparations pre-treated with 500 µg reserpine (top traces: initial response; lower traces: expanded excerpts, as in Fig. 1). Scale bars, A,B 100 µm; C,D 10 s upper traces, 100 ms lower traces.

2000; Allen and Murphey, 2007), none are part of the flight system. Future studies must verify whether TCG, and other interneurones that initiate flight (cf. Pearson et al., 1985; Ramirez, 1988), or other rhythmic motor patterns (cf. Heinrich, 2002) are cholinergic.

Amine depletion

To evaluate the necessity of amines for flight initiation we treated locusts with reserpine at over twice the dosage that depletes amines from insect nervous tissue to levels below detection by radiochemical assay and HPLC (cf. Robertson, 1976; Sloley and Owen, 1982) and checked its efficacy by immunocytochemistry (Fig. 6). Although lethargic, reserpinized locusts still generate a normal flight motor pattern in response to wind stimulation (Fig. 2 and Fig. 6C, Table 1). Likewise, amine-depleted crickets (Stevenson et al., 2000), and octopamine-deficient *Drosophila* mutants still produce flight movements (Monastirioti et al., 1996), though less readily (Brembs et al., 2007). Furthermore, in our hands, the specific octopamine antagonist epinastine and the alpha-adrenergic receptor antagonist phentolamine failed to block flight initiation selectively and reversibly at concentrations shown to be effective in other systems (cf. Roeder et al., 1998; Stevenson et al., 2005). Conversely, the muscarinic receptor antagonists atropine and scopolamine reversibly blocked flight initiation by octopamine (Fig. 5F), whereas pilocarpine still initiated fictive flight in reserpinized locusts (Fig. 6D). This is in accordance with the findings that flight-initiating neurones are distinct from octopaminergic (Stevenson and Spörhase-Eichmann, 1995) and dopaminergic neurones (Watson, 1992), and that identified octopaminergic neurones in thoracic ganglia do not initiate flight (Libersat and Pflüger, 2004). We conclude that amines are not essential for locust flight initiation and that muscarinic activation of flight is not dependent on biogenic amines, whereas aminergic flight initiation requires muscarinic receptors.

Aminergic effects

So what role do amines play in flight initiation? Some role is suggested since several different amines (Fig. 5), but not serotonin or the precursor amino acid tyrosine, can initiate flight. The effective concentrations of amines for flight release were extraordinarily high, so that non-specific activation of related receptors may occur. For example, the trace amines noradrenaline and adrenaline lack dedicated receptors in insects (cf. Roeder, 1994; Roeder, 2005) but nonetheless induced flight at a concentration of 500 mmol l^{-1}. Since the most effective flight initiating concentration for octopamine was equally high (500 mmol l^{-1}), its action may also be non-specific. Dopamine was at least as effective as octopamine, except that the flight sequences were interrupted by pauses. Dopamine also initiates flight in moths (Claassen and Kammer, 1986) and locomotor activity in *Drosophila* (Yellman et al., 1997; Andretic et al., 2005; Kume et al., 2005). Histamine, which is present in thoracic ganglia (Hörner et al., 1996) but has no known function there, effectively initiated flight at even lower dosages (10 mmol l^{-1}), but here the delay to flight onset took minutes rather than seconds. Tyramine,

the precursor of octopamine and an insect neurotransmitter candidate (for reviews, see Roeder, 2005; Scheiner et al., 2006), generally has opposing effects to octopamine (Saraswati et al., 2004; Fox et al., 2006; Fussnecker et al., 2006) and is claimed to inhibit flight initiation in *Drosophila* (Brembs et al., 2007). In our hands, however, tyramine was the most effective flight-initiating amine, acting equally and as rapidly as octopamine, but at a significantly lower concentration (10 mmol l^{-1}; Fig. 2, Table 1). We speculate that tyramine acts selectively, since it only binds to octopamine receptors at 100-fold concentration (Balfanz et al., 2005). In conclusion, tyramine, dopamine and histamine must all be considered, in addition to octopamine, as potentially influencing the flight CPG.

Octopamine enhances numerous behavioural responses in insects (Stevenson and Kutsch, 1987; Weisel-Eichler and Libersat, 1996; Stevenson et al., 2005), and facilitates cholinergic synaptic transmission in the cockroach escape circuit (Casagrand and Ritzmann, 1992b) and the response of TCG to wind stimulation (Ramirez et al., 1989) (personal observations). For flight released by the amines we consistently found larger values for the elevator phase in the depressor cycle (Fig. 2C, Table 1). Accordingly, we suggest that amines, rather than acting as neurotransmitters to initiate flight, act as neuromodulators to facilitate the cholinergic flight-initiating pathway and shape the final motor pattern. This could be adaptive, ensuring that specific releasing stimuli elicit flight under behaviourally relevant circumstances, and may be a general feature of behavioural control in insects. Analogous pathways may operate in vertebrates, and possibly humans (Hultborn and Nielsen, 2007), where locomotion is initiated by amino acids (e.g. Douglas et al., 1993) and facilitated (e.g. Beninger, 1983; Grillner et al., 2005) or modulated (e.g. Barbeau and Rossignol, 1991) by monoamines. Future studies on insects must determine which amines are specifically involved and how they interact with cholinergic transmission.

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