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Electrical activity of caudal neurosecretory neurons in seawater and freshwater-adapted *Platichthys flesus*, *in vivo*

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Summary

This study examined the electrical firing activity of neuroendocrine Dahlgren cells in the caudal neurosecretory system (CNSS) of the euryhaline flounder *in vivo*. Intracellular recordings revealed generally similar activity patterns and membrane properties to those previously reported *in vitro*. To investigate the potential role of the CNSS in osmoregulatory adaptation, extracellular, multiunit, recordings compared the activity patterns of Dahlgren cells in fully seawater- and freshwater-adapted fish. The proportion of cells showing bursting (as opposed to phasic or tonic) activity was greater in seawater- than in freshwater-adapted fish, as was the Correlation Index, a measure of the degree of correlation between firing activities of cells recorded simultaneously from the same preparation. Acute transfer of fish from seawater to freshwater gill perfusion led to recruitment of previously silent Dahlgren cells and a reduction in Correlation Index; freshwater to seawater transfer increased the Correlation Index. Severing the spinal cord anterior to the CNSS led to an increase in overall Dahlgren cell activity. Electrical stimulation of branchial nerve branches providing input to the brainstem, or tactile (pinch) stimulation of lips or fins, led to a reduction in CNSS activity lasting up to 500 s, indicating the presence of descending modulatory pathways from the brain. These results are consistent with a role for CNSS neuropeptides, urotensins, in supporting survival in a hypertonic, seawater, environment.

Key words: flounder, *Platichthys flesus*, neurosecretion, Dahlgren cell, osmoregulation, electrical bursting activity

Introduction

The caudal neurosecretory system (CNSS) is a discrete neuroendocrine system, structurally unique to fish. It comprises a population of several hundred magnocellular neurosecretory Dahlgren cells located in the last 6–8 terminal segments of the spinal cord, whose axons project caudally to the neurohaemal organ or urophysis (review by Winter et al., 2000). Thus the system has organisational parallels with the vertebrate neurohypophysial neuroendocrine system, with the added benefit of accessibility for both *in vitro* and *in vivo* study. Neuropeptides synthesised and secreted by the CNSS, specifically urotensins I and II (UI and UII), have been shown to play a role in osmoregulatory adaptation (Bern et al., 1985; Lederis et al., 1985; Winter et al., 2000). This is particularly important in euryhaline fish, such as the flounder, *Platichthys flesus*, which is capable of full adaptation to seawater (SW) and freshwater (FW) environments. Both UI (L. Dow and R. J. Balment, unpublished) and UII (Winter et al., 1999) show altered urophysial (UI, UII) content and plasma (UII) concentrations, when animals are transferred between FW and SW, indicating altered neuroendocrine synthesis and secretion.

Electrophysiological recording from an isolated CNSS preparation, comprising spinal cord and urophysis *in vitro*, indicated the presence of two types of Dahlgren cell (Hubbard et al., 1996a; Brierley et al., 2003). Type 1 (T1) cells are spontaneously active with activity patterns ranging from tonic, through phasic, to the generation of characteristic bursting, reminiscent of that reported for mammalian vasopressin neurons (Leng et al., 1999). Type 2 (T2) cells are silent and relatively inexcitable; they exhibit strong spike frequency accommodation and fire only a single action potential in response to a long duration depolarising stimulus. Otherwise, membrane properties of the two cell types are similar, apart from a significantly smaller spike afterhyperpolarisation (AHP) in T2 compared with T1 cells (Brierley et al., 2003). Variations in firing patterns are likely to be related to differential neuropeptide secretion from the urophysis and we might therefore expect to see differences in electrical activity of Dahlgren cells under different osmoregulatory conditions.

We have previously compared spontaneous firing patterns and membrane properties of Dahlgren cells in isolated (*in vitro*) CNSS taken from flounder fully adapted to either SW or FW (Brierley et al., 2003). This revealed few differences, apart from apparently less robust bursting activity in FW-adapted cells. However, this preparation lacks descending modulatory

input, which may carry information about the fish's internal and external osmotic environment. The role of such descending input is suggested by pharmacological investigations, which identified a range of potential neuromodulators in this system, including noradrenaline, serotonin and acetylcholine (Hubbard et al., 1996b, 1997; Brierley et al., 2003).

The aim of this study was initially to determine whether electrophysiology and activity of Dahlgren cells *in vivo* are comparable with those previously reported *in vitro*. We then aimed to test the hypotheses that: first, SW–FW adaptation is associated with differences in electrical activity patterns of Dahlgren cells (and hence peptide secretion) *in vivo*; and, second, activity in the CNSS is modulated by descending inputs from the brain. We first compared intrinsic membrane properties and firing patterns of Dahlgren cells between SW- and FW-adapted preparations *in vivo,* and between *in vivo* and *in vitro* preparations. We then carried out initial studies to examine effects of manipulation of central pathways, including those potentially involved in signalling changes in osmolarity of the external medium.

Materials and methods

Adult flounder, *Platichthys flesus* L. (350–700 g) were caught in Morecambe Bay or the Dee Estuary (UK) and held in aquaria under a 12:12 h light:dark cycle in either seawater (SW, 860–1000 mOsmol^{-1}) or freshwater (FW, 0–6 mOsmol^{-1}) at 10°C, for at least 14 days prior to experimentation.

Fish were anaesthetised as detailed under UK Home Office licensing procedures. They were immersed in MS222 (ethyl-m-aminobenzoate methane sulphonate, 100 mg l^{-1}) or Benzocaine (ethyl-p-aminobenzoate, 50 mg l^{-1}) (Sigma, UK). After 4–5 min fish became sedated, were weighed and then given an intraperitoneal injection of Saffan (alphaxalone/alphadalone acetate mixture, 36 mg kg^{-1} body weight, Schering-Plough Animal Health, UK). This produces long term (4–8 h), stable anaesthesia in teleosts (Oswald, 1978). During induction of surgical anaesthesia, the regular opercular rhythm associated with gill ventilation gradually disappeared. The fish was placed on a platform, covered in damp paper towel and immobilised to stabilise the caudal region for surgery and recording. The gills were ventilated by continuous perfusion of water (SW or FW, 1000 ml kg^{-1} body weight min^{-1}) through the buccal cavity and over the gills *via* a centrifugal pump (Type 1250, Eheim Ltd, Germany). Water was recirculated through a feeder tank (20 l volume), where it was aerated and cooled to 7–8°C. During surgery and recording, the general physiological state of the fish was continuously monitored by checking the normal red colour of the gills and by monitoring the electrocardiogram (ECG) using small metal electrodes on the skin surface. Heart rate was typically 30–50 beats min^{-1}. Fish were maintained under stable anaesthesia for up to 8 h.

A 2.5–3.0 cm incision was made adjacent to the lateral line in the caudal region and the muscle overlying the spinal column retracted. Bone covering preterminal spinal cord segments 2–5 was drilled or clipped away and the meningeal sheath surrounding the cord removed using fine forceps. Accumulation of tissue fluid within the surgical site caused blockage of intracellular microelectrodes, so a Ringer perfusion/extraction system was established. Ringer was designed to match plasma ion levels for flounder adapted to SW or FW. Seawater Ringer composition was (in mmol l^{-1}): K_2HPO_4 1.0, KCL 0.5, NaCl 155, $NaHCO_3$ 10, $CaCl_2$ 2.12, $MgSO_4$ 1.0, D-glucose 5.56, adjusted to pH 7.7 (osmolality 315 mOsmol kg^{-1} H_2O). Freshwater Ringer had a NaCl concentration of 145 mmol l^{-1} and osmolality 280 mOsmol kg^{-1} H_2O. Ringer was cooled to 3–4°C and then perfused (1.0 ml min^{-1}) over the exposed spinal cord *via* a peristaltic pump (Type 302S, Watson Marlowe Ltd, UK). This maintained the viability of the tissue where local blood vessels were disrupted when the meningeal sheath was removed for intracellular recording. The temperature at the recording site, monitored using a small thermocouple and electronic thermometer, was 10–12°C and Ringer was not recirculated.

Intracellular recording

Intracellular recordings were made from Dahlgren cells using glass microelectrodes pulled on a micropipette puller (Model 750, David Kopf Instruments, CA, USA) and filled with 3 mol l^{-1} potassium acetate (resistance 50–100 MΩ). The electrode was connected to an Axoclamp 2A amplifier (Axon Instruments, CA, USA) and data captured (sampling rate 5 kHz) *via* a CED 1401 converter (Cambridge Electronic Design, UK). Data were stored and subsequently analysed using CED Signal (v1.72) or Spike2 (v2.02) software. Following cell penetration, the electrode was bridge balanced using 500 ms –0.3 nA current pulses at 0.3 Hz. Only cells that maintained a stable resting potential more negative than 50 mV and could generate overshooting action potentials were considered viable.

Extracellular recording

Recordings were made from preterminal segments 2–3 using a glass suction electrode (diameter 300–600 μm) (Brierley et al., 2003). Signals were recorded differentially and amplified using a Neurolog (Digitimer, UK) AC NL104 amplifier with NL 100K probe (headstage), filtered (NL125, 5 Hz and 1.25 kHz cut off and a 50 Hz notch filter), and digitised through a CED micro 1401. Spike2 (v4.09) software was used for data capture (sampling rate 8 kHz) and off-line analysis. This software enables separation of activity of individual units, by generating an action potential template for each unit and scanning the recording for similar waveforms. Action potentials from different units are then displayed as event marks on separate traces.

Acute salinity exchange

Separate 20 l tanks of SW and FW were cooled to 7–8°C and water pumped through appropriately adjusted flow-meters to a manually operated two-way valve. Seawater or FW could

then be selected as required to supply the fish mouthpiece. Water draining from the gill covers was returned to the appropriate tank for cooling and recirculation. The system was designed to minimise mechanical disruption of the recordings during switching.

Nerve stimulation

The branchial branches of the glossopharyngeal and vagal cranial nerves were exposed by dissection near to their roots in the brainstem medulla and supported in a liquid paraffin pool on a pair of 0.2 mm diameter, stainless steel hook electrodes for stimulation. Electrically isolated constant voltage stimuli were produced by a S88C stimulator and SIU5 isolator (Grass Instrument Co., USA) and consisted of 1 s trains of 0.2 ms duration pulses at 20 Hz, with voltages in the range 10–40 V.

Results

Intracellular recording

Type 1 and T2 Dahlgren cells were recorded *in vivo* from CNSS of both SW- and FW-adapted fish. As for *in vitro* studies (Hubbard et al., 1996a), T1 cells were usually spontaneously active. Type 2 cells were typically silent and were recorded less frequently: only 14% of cells penetrated. The cell types were distinguished by the marked difference in amplitude and duration of spike afterhyperpolarisation (AHP), which was much smaller in T2 cells, and by the strong spike frequency accommodation shown by T2 cells; T1 cells fired repeatedly in response to depolarising current injection whereas T2 cells fired only once. Fig. 1A shows typical responses of these cells to a depolarising current pulse.

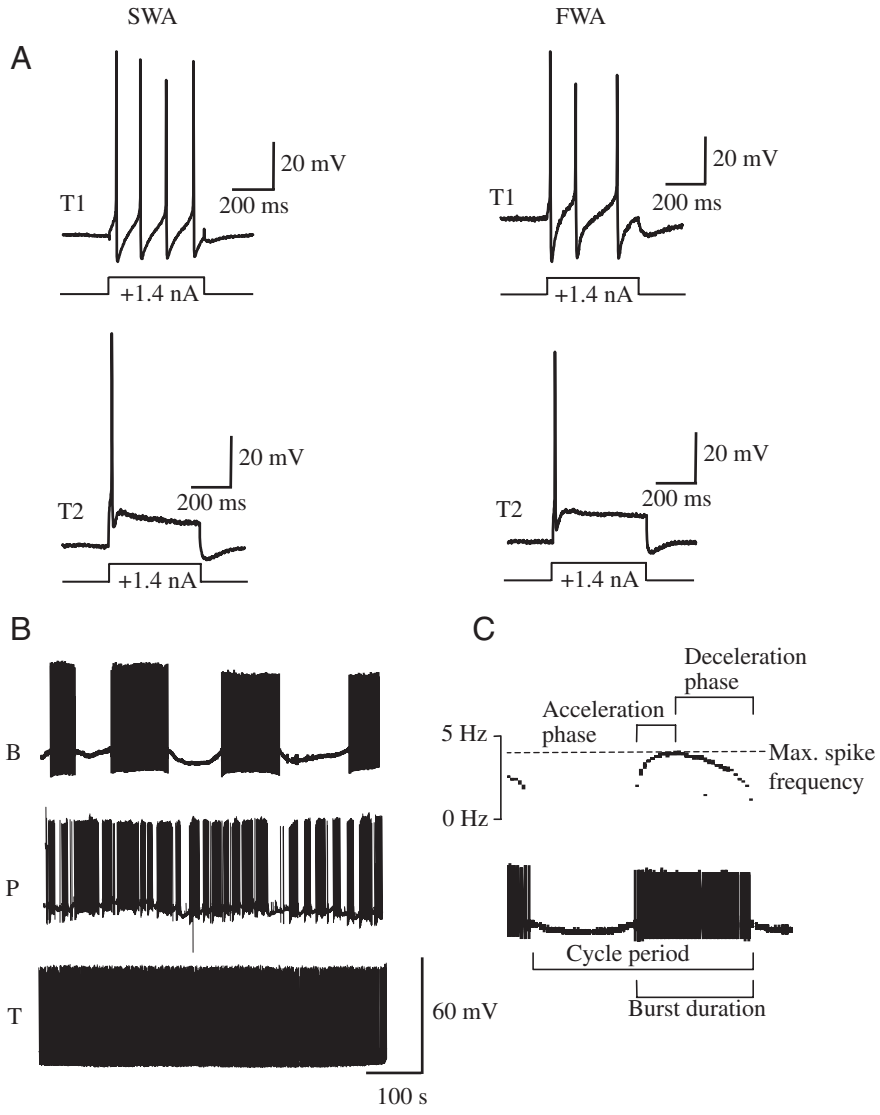

Fig. 1. Activity of T1 and T2 Dahlgren cells in SW- and FW-adapted flounder, recorded intracellularly. (A) Responses to depolarising current injection (1.4 nA, 500 ms) are similar in SW and FW-adapted fish. Note large amplitude AHP in T1 and characteristic spike frequency accommodation in T2. (B) Activity patterns in T1 cells (all SW-adapted): B, typical spontaneous bursting; P, phasic; T, tonic. (C) Single burst recorded from a Dahlgren cell with instantaneous spike frequency plot (top), showing how burst parameters were defined.

For comparison with previous work on isolated CNSS (Hubbard et al., 1996a; Brierley et al., 2001, 2003), the membrane properties of T1 and T2 cells *in vivo* were analysed further. Table 1A presents membrane and action potential parameters for T1 cells from fully SW- and FW-adapted fish. There were no significant differences between the two adaptation states for any of these, suggesting no difference in underlying membrane conductances. Although relatively few T2 cells were encountered, similar data are presented for T2 cells (Table 1B). Again, there were no obvious differences in membrane parameters between SW- and FW-adapted fish. As expected from previous studies *in vitro* (Hubbard et al., 1996a; Brierley et al., 2003), significant differences were found both in action potential threshold and in AHP duration of T2 compared with T1 cells (SW-adapted) (Table 1).

As reported by Brierley et al. (2001, 2003) for Dahlgren cells *in vitro*, spontaneous activity in T1 cells *in vivo* varied between tonic, phasic and bursting activity patterns (Fig. 1B). Activity patterns were classified as defined by Brierley et al. (2003): ‘bursting’, comprising discrete >20 s bursts of action potentials separated by periods of >20 s quiescence; ‘phasic’, showing irregular activity including periods of high (1–3 Hz) and low (<1 Hz) frequency firing; and ‘tonic’, consisting of

Table 1. *Membrane and action potential parameters of (A) T1 and (B) T2 Dahlgren cells from SW- and FW-adapted fish* in vivo

(A) T1 membrane parameter	SW-adapted *N*=18	FW-adapted *N*=15
Resting membrane potential (mV)	–62.4±1.3	–62.9±1.6
Input resistance (MΩ)	25.4±1.7	27.9±1.4
Action potential threshold (mV)	–53.7±1.7	–53.2±1.5
Action potential amplitude (mV)	78.1±2.6	81.6±2.4
Action potential duration (ms)	3.5±0.2	3.6±0.2
Afterhyperpolarisation amplitude (mV)	12.9±0.9	13.6±1.0
Afterhyperpolarisation duration (ms)	198.8±13.8	219.7±22.8

(B) T2 membrane parameter	SW-adapted *N*=3	FW-adapted *N*=2
Resting membrane potential (mV)	–65.0±0.9	–74, –60
Input resistance (MΩ)	25.7±6.7	46.0, 28.2
Action potential threshold (mV)	–42.9±1.5*	–42.7, –39.5
Action potential amplitude (mV)	76.7±1.5	61, 76
Action potential duration (ms)	3.8±0.4	3.5, 3.2
Afterhyperpolarisation amplitude (mV)	9.4±2.5	8.1, 9.5
Afterhyperpolarisation duration (ms)	32.3±3.2*	34, 15

Data are mean ± S.E.M. For FW T2, raw data are presented for two individual cells.

*P<0.05 (Student's t-test) compared with T1.

Table 2. *Comparison of burst parameters from spontaneously bursting T1 Dahlgren cells recorded* in vivo *(A), and* in vitro *(B), from SW- and FW-adapted flounder*

(A) *In vivo*: burst parameter	SW-adapted *N*=8	FW-adapted *N*=7
Duration (s)	54.6±9.4	89.9±14.1
Cycle period (s)	161.1±33.1	212.4±38.7
Acceleration phase (s)	14.7±4.4	21.8±4.3
Deceleration phase (s)	39.9±6.4	67.3±11.3*
Number of spikes per burst	172±39	272±50
Maximum spike frequency (Hz)	5.7±1.0	5.1±0.7
Mean spike frequency (Hz)	3.5±0.9	3.2±0.3

(B) *In vitro*: burst parameter	SW-adapted *N*=14	FW-adapted *N*=8
Duration (s)	124.0±13.8†	89.9±22.4
Cycle period (s)	382.2±49.4†	278.3±56.2
Acceleration phase (s)	28.7±3.5†	23.1±7.6
Deceleration phase (s)	95.2±11.7†	66.8±15.3
Number of spikes per burst	324±40†	177±38*
Maximum spike frequency (Hz)	4.5±0.4	3.6±0.5
Mean spike frequency (Hz)	2.6±0.2	2.2±0.3†

Data are mean ± S.E.M.

*P<0.05 (Student's t-test) compared with SW. †P<0.05, compared with *in vivo*. Data in B taken from Brierley et al. (2001; M. J. Brierley, unpublished).

continuous activity ~1 Hz with few or no silent periods. There was no obvious difference in the range of activity patterns of cells recorded intracellularly from SW- and FW-adapted fish. Burst parameters were calculated for bursting T1 cells (Fig. 1C) to highlight differences between SW- and FW-adapted states (Table 2A). The trend was towards longer burst duration and cycle period in FW compared with SW fish. However, these differences were not statistically significant, possibly owing to substantial variation between cells. The only parameter that showed a difference was the deceleration phase (time from peak firing rate to the end of a burst), which was significantly longer in cells in FW-adapted fish, suggesting less tight control over burst termination than in SW cells.

Table 2B presents comparable data, taken from Brierley et al. (2001; M. J. Brierley, unpublished), for bursting T1 cells recorded intracellularly in the *in vitro* (isolated) CNSS preparation. In contrast to cells recorded *in vivo*, there was a trend towards shorter bursts in cells from FW- compared with SW-adapted preparations, which was reflected in significantly fewer spikes per burst. Statistical comparison between burst parameters of cells recorded *in vivo* and *in vitro* revealed significant differences (Table 2). In SW-adapted fish, burst duration and cycle period were significantly longer (P<0.01; Student's t-test) and the number of action potentials per burst significantly greater (P<0.05) *in vitro* than *in vivo*; maximum and mean firing frequency within bursts did not differ. In FW fish, bursts were similar between the two preparations; there were no significant differences between burst parameters recorded *in vitro* compared with *in vivo*, apart from a lower (P<0.05) mean firing frequency within bursts *in vitro* (Table 2B).

Extracellular recording

Spike2 analysis allowed separation of activity of up to 14 Dahlgren cells from each fish, yielding a total of 56 cells from five SW-adapted and 69 cells from five FW-adapted fish. Dahlgren cells were clearly identified by their long duration action potentials (Brierley et al., 2003). Action potential duration at half spike amplitude was 2.5±0.5 ms for SW and 2.5±0.4 ms for FW Dahlgren cells. Cells with extracellular spike duration less than 1.8 ms were not included in the analysis. These were probably α neurons with markedly briefer spikes (<1 ms; Brierley et al., 2003). Action potential amplitude was 0.29±0.14 mV for SW and 0.31±0.12 mV for FW Dahlgren cells. Fig. 2 shows a recording from a SW-adapted fish, showing total activity and that of 10 individual units with different activity patterns.

To identify any differences in spontaneous activity patterns of Dahlgren cells between SW- and FW-adapted fish, extracellular recordings were taken for 2000 s and then analysed. Each unit was classified for its firing pattern: tonic, phasic, bursting, as defined by Brierley et al. (2003). (Silent cells, by definition, were not recorded.) The total number of

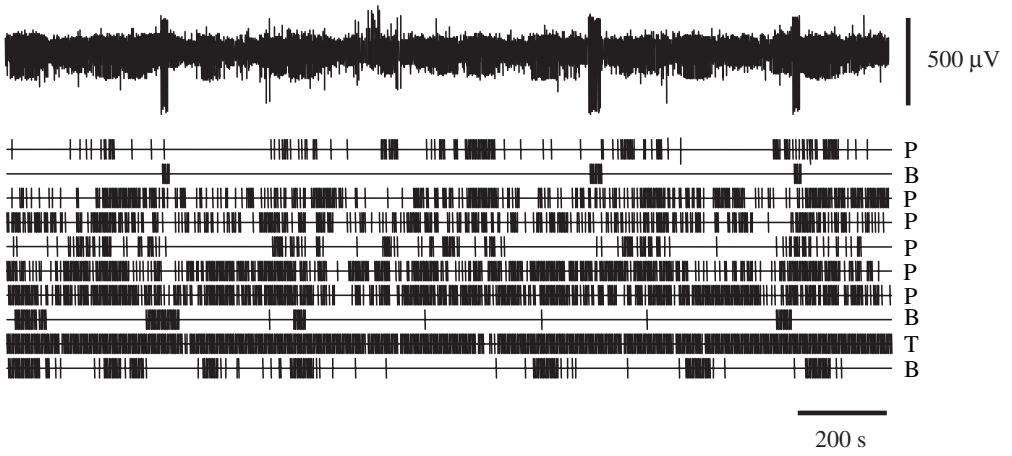

Fig. 2. Extracellular multiunit recording from CNSS of SW-adapted flounder *in vivo*, showing raw trace (top) and activity of ten individual units separated by off-line Spike2 analysis. Dahlgren cells show a range of firing activity patterns, including: T, tonic; P, phasic; B, bursting.

spikes generated (per 1000 s) was used as a measure of overall activity. The degree of 'patternedness' of cells' firing activity was assessed by sorting the spike data into 50 s time bins and measuring the maximum and minimum number of spikes per bin. Table 3 summarises these data for all units from SW and FW adapted fish. Cells were almost all classified as either bursting (28% SW; 16% FW) or phasically active (72% SW; 82% FW), with the majority the latter. Furthermore, the proportion of cells classified as bursting in SW-adapted CNSS (28%) was significantly (P<0.05; χ^2-test) greater than in FW-adapted (16%) fish. There was no significant difference in any other measured parameter between the two adaptation states. Overall activity was highly variable between cells, ranging from less than 20 to more than 2000 spikes per 1000 s. However, the mean of these rates did not differ between SW and FW. The maximum firing frequency was around 5 Hz, as reported *in vitro* (Brierley et al., 2001). Minimum and maximum firing rates per 50 s time bin were similar for the two adaptation states.

Table 3. *Summary of spontaneous firing activity of Dahlgren cells recorded extracellularly from SW- (*N=*5) and FW-adapted (*N=*5) flounder*

	SW-adapted *N*=56	FW-adapted *N*=69
Firing pattern		
Bursting (%)	28	16*
Phasic (%)	72	82
Tonic (%)	0	2
Spikes per 1000 s		
Range	22–1527	15–2235
Mean ± S.D.	342±362	374±427
Maximum firing frequency		
Mean ± S.D. (Hz)	5.0±2.7	5.3±3.0
Maximum spikes per 50 s bin		
Range	3–218	3–213
Mean ± S.D.	66.8±56.5	56.2±41.8
Minimum spikes per 50 s bin		
Range	0–30	0–13
Mean ± S.D.	2.0±5.1	1.3±2.6

*P<0.05 (χ^2 test) compared with SW.

Flounder were exposed to acute change in salinity of water perfusing the gills during continuous extracellular recording from the CNSS. Three SW- and four FW-adapted flounder were recorded for 2000 s and then the gill perfusion was changed to FW and SW, respectively, followed by further recording for 2000 s. Firing activity was compared pre- and post-water change. No overall changes in total firing activity, maximum or minimum firing rates of Dahlgren cells were observed. However, some cells did change their firing pattern, including a number that were either recruited (silent in control) or whose activity was abolished (became silent) following water change. Table 4 shows the proportion of cells showing the different firing patterns. It was possible to include silent cells in this analysis, but only those that showed a change in activity (from silent to active or *vice versa*) following water change. A change from SW to FW led to a significant (P<0.05;

Table 4. *Proportions of Dahlgren cells showing different activity patterns before and after transfer from SW to FW and from FW to SW perfusing the gills*

	Before	After
SW→FW (*N*=34 cells from three fish)		
Bursting (%)	23	20
Phasic (%)	68	57
Tonic (%)	0	0
Silent (%)	9	23*
FW→SW (*N*=58 cells from four fish)		
Bursting (%)	17	20
Phasic (%)	67	71
Tonic (%)	2	0
Silent (%)	14	9

*P<0.05 (χ^2 test) compared with before.

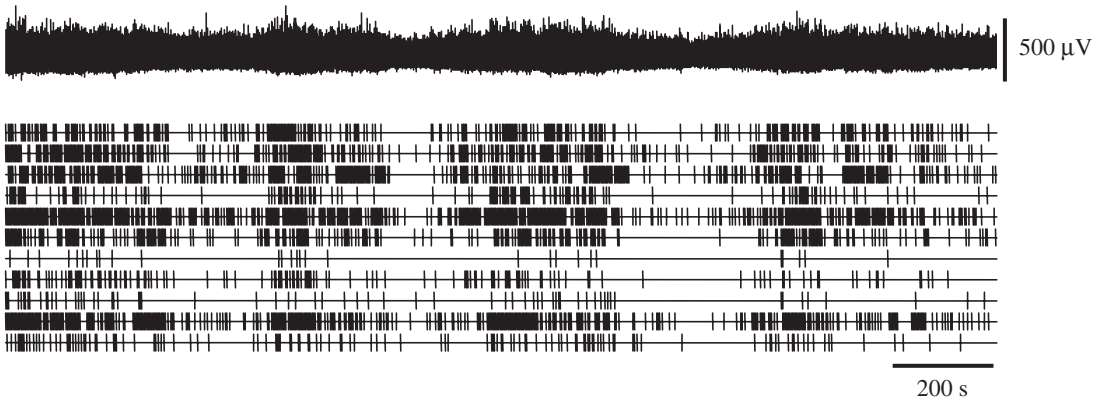

Fig. 3. Extracellular multiunit recording from CNSS of SW-adapted flounder *in vivo*, showing apparent correlation of activity in 11 different units (separated by Spike2 analysis), which combines to reveal periodic increases in overall activity in the raw trace (top).

χ^2-test) increase in the number of silent cells, from 9 to 23%. Conversely, the FW to SW switch led to an apparent reduction in silent cells, though this was not significant. The proportion of cells generating other activity patterns was unchanged following the salinity switch.

Visual inspection of recordings suggested that firing activity of different units within a preparation was often correlated (e.g. Fig. 3). In order to quantify this, a Correlation Index (CI) was calculated for CNSS of each individual fish both before (2000 s) and after (2000 s) the switch in gill perfusion (Table 5). For each unit, spikes were sorted into 20 s time bins. Cross correlation was used to compare each unit with all the others for each time bin and assessed for significance using Pearson correlation (two-tailed; significance assumed at $P<0.05$). The CI was defined as the proportion of the total comparisons for each CNSS that showed significance. Correlation Index ranged from 0.06 to 0.94 and was significantly ($P<0.05$, unpaired) greater in SW compared with FW fish. Furthermore, a switch from SW to FW led to a significant reduction in CI ($P<0.05$, paired), whereas the switch from FW to SW caused a significant increase in CI ($P<0.05$, paired).

Table 5. *Correlation indices (CI) for CNSS of SW- (*N=*3) and FW-adapted (*N=*4) flounder before and after transfer to FW and SW, respectively, perfusing the gills*

	Correlation index	
Fish	Before	After
SW→FW		
22/8	0.85	0.56
11/9	0.94	0.62
30/11	0.86	0.76
FW→SW		
10/8	0.06	0.35
13/8	0.67	0.85
11/10	0.46	0.52
31/10	0.46	0.79

Cross correlation was used to compare the activity (within 20 s time bins) of each unit with all the others. The CI was defined as the proportion of the total comparisons for each CNSS that showed significance ($P<0.05$, Pearson correlation).

In 17 fish (nine SW-, eight FW-adapted), at the end of an experiment, the spinal cord was severed one or two segments anterior to the recording site. In 12 cases (five SW, seven FW), this resulted in a marked increase in the firing activity of Dahlgren cells (Fig. 4). A similar response was obtained from one fish (SW-adapted) in which the cord was cut much higher up, at its junction with the posterior hindbrain. The response to cutting the cord was characterised by an initial burst of irregular large amplitude (1–2 mV) spikes lasting for ~10 s. This was followed by a prolonged period (up to 20 min) of increased overall activity, mainly owing to recruitment of previous silent units, many of which were bursting (Fig. 4). Preliminary experiments were also carried out to identify any responses in the CNSS following stimulation of input pathways to the brain. Electrical stimulation of the branchial branches of the glossopharyngeal and/or vagal nerves (1 s train, 20 Hz, 0.2 ms, 40 V) led to a long lasting (up to 400 s) change in Dahlgren cell firing, comprising an overall reduction in activity (Fig. 5A). Tactile (mild pinch) stimulation of lips and fins also induced marked changes in activity in some preparations, lasting up to 500 s and consisting of an initial period of inhibition (~150 s) followed by a gradual return to the control activity level, and sometimes including recruitment of previously silent units (Fig. 5B,C). These results imply a strong descending influence over Dahlgren cell activity.

Discussion

Electrophysiological properties of Dahlgren cells recorded intracellularly *in vivo* were remarkably similar to those *in vitro,* previously reported (Hubbard et al., 1996a; Brierley et al., 2001, 2003). Both T1 and T2 Dahlgren cells were identified, including characteristic differences between the two of marked spike frequency accommodation of T2 in response to depolarising input, and smaller amplitude AHP in T2 compared with T1.

Overall, the range and nature of spontaneous firing patterns recorded from T1 were similar *in vivo* compared with *in vitro*, including burst durations, cycle periods and a maximum firing frequency around 5 Hz. This suggests that, for these electrophysiological parameters at least, the *in vitro* preparation can provide a model for investigating the relationship between electrical activity and peptide synthesis and secretion. However, several significant differences were observed between SW-adapted bursting cells in the two types of preparation. Both cycle period and burst duration were shorter *in vivo* compared with *in vitro*, and this was accompanied by a greater number of spikes per burst in the latter. These differences may be due to descending influences on intrinsic burst generation mechanisms, which are removed in the *in vitro* preparation. Thus, it is clear that recordings made *in vitro* do not provide an exact reflection of activity that occurs *in vivo*. Similar differences were not found for FW-adapted fish. Since burst duration and frequency depend, at least in part, on mechanisms intrinsic to the Dahlgren cells, it is possible that these mechanisms are less susceptible to descending modulation in the FW-adapted state.

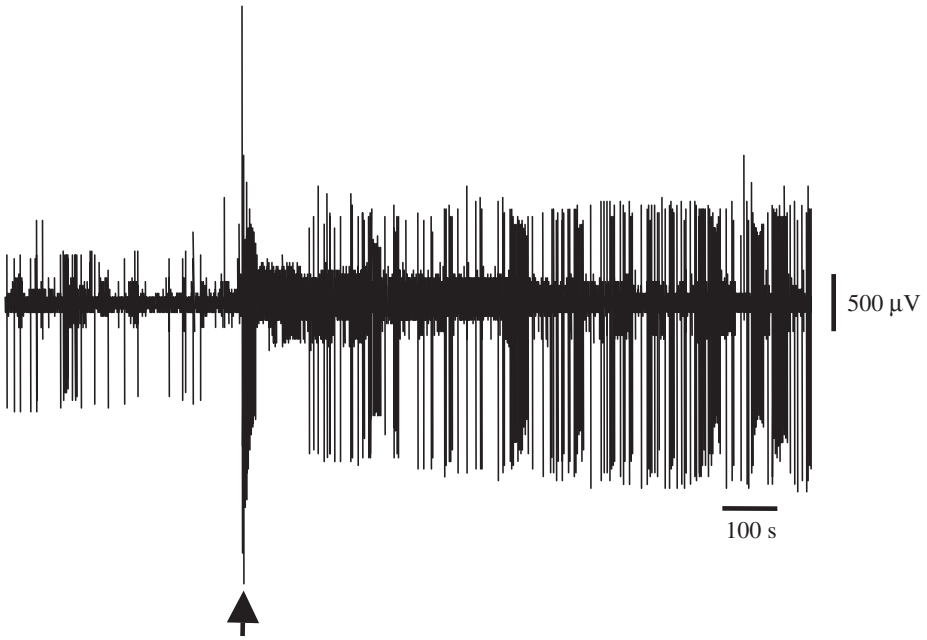

Fig. 4. Extracellular multiunit recording from CNSS of SW-adapted flounder. Cutting the spinal cord just above the recording site (arrow) led to increased activity including recruitment of previously silent units and enhanced bursting activity.

One interesting difference between *in vivo* and *in vitro* multiunit data was a lower proportion of cells exhibiting bursting or tonic, as opposed to phasic, activity patterns in the former. Brierley et al. (2003) reported 60–65% bursting, 20% phasic and 15–20% tonically active cells recorded extracellularly *in vitro*. By contrast, in this study 72–82% of cells were phasic with only 16–28% bursting and 0–2% tonic. Presumably this was again due to the absence of descending modulatory input in the isolated *in vitro* CNSS preparation, supporting the view that bursting activity is an intrinsic emergent property of the CNSS and that extrinsic (descending) modulation disrupts this pattern, having the overall effect of making Dahlgren cells fire more irregularly (phasic activity).

When comparisons were made between T1 cells recorded from chronically SW- *vs* FW-adapted fish *in vivo*, there were no differences in resting membrane properties or action potential generation. However, bursting cells in FW-adapted fish showed an apparently longer burst duration, which was due to a significantly longer deceleration phase. A similar finding was reported *in vitro* where bursts in FW-adapted cells were more variable in duration than their SW counterparts (Brierley et al., 2003), suggesting less tight control over burst duration and termination in the FW state.

Although firing activity was similar in T1 Dahlgren cells from chronically SW- and FW-

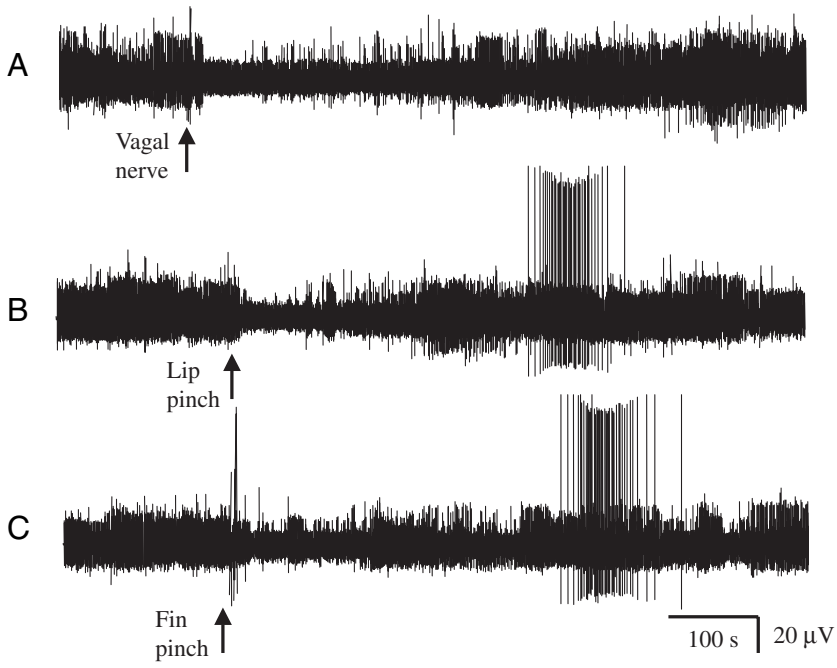

Fig. 5. Extracellular multiunit recording from CNSS of SW-adapted flounder, showing response to stimulation of branchial branch of the vagal nerve (A, arrow), and mild pinch to lip (B) or fin (C). All three treatments led to an overall reduction in activity lasting around 400 s.

adapted fish, the proportion of cells showing different activity patterns was not. Significantly more cells were bursting in SW-adapted fish, again supporting the view that bursting is less robust in the FW-adapted state. Similarly the Correlation Index was greater in SW preparations and showed a significant reduction following acute transfer to FW, with a corresponding increase for the reverse transfer (FW to SW). The acute switch to FW in chronically SW-adapted fish also led to a significant increase in the number of silent Dahlgren cells (i.e. de-recruitment).

Bursting activity in Dahlgren cells depends on intrinsic cellular mechanisms including Ca-mediated depolarising after potentials (DAP) and post-burst after depolarisation (ADP), which maintain repetitive firing and underlie prolonged burst generation respectively (Brierley et al., 2001, 2003, 2004). However, the significant CIs found here also suggest co-ordination of cellular firing within the population. We have no evidence that Dahlgren cells are directly synaptically linked (Winter et al., 2000), so that co-ordination within the population is most likely mediated either by patterned descending input or by rhythm generation intrinsic to the CNSS, involving either a presynaptic interneuronal network or release of an intrinsic neuromodulator. With respect to the latter, nitric oxide (NO) has been implicated as a local neuromodulator in this system. Dahlgren cells in another teleost, *Oreochromis niloticus*, express nitric oxide synthase (Cioni et al., 1997) and in flounder we have shown that they are excited by application of a NO donor (Brierley et al., 2002). Thus, it is possible that Dahlgren cell activity is coordinated by local positive feedback, in a similar way to the synchronised high frequency bursts of oxytocin neurons (Russell et al., 2003). The functional relevance of coordinated activity between Dahlgren cells is probably related to the regulation of peptide secretion. Bursts of spikes in neuroendocrine cells are known to enhance peptide secretion, when compared with tonic firing (Cazalis et al., 1985). The degree of synchronisation of the timing of these bursts in a neuron population (i.e. CI) could potentially allow for a continuum of secretory pattern, ranging from continuous low level secretion (no synchrony) through to highly pulsatile release. Similarly, multiunit recordings from populations of immortalised GnRH neurons (Nunemaker et al., 2001) revealed episodes of increased firing activity within the population, which are due to co-incident activity in subpopulations of neurons, and which appear to underlie episodic (pulsatile) GnRH secretion. It was suggested that variations in the number of units contributing to such episodes could account for changes in GnRH pulse amplitude in this system (Nunemaker et al., 2001).

The response to cutting the spinal cord was excitatory, suggesting the presence of a descending continuous tonic inhibitory drive to the CNSS. The initial brief excitatory burst (10 s) was probably due to activation of excitatory fibres as they were cut. The longer term response included recruitment of units and enhanced bursting activity, again supporting the hypothesis that bursting activity is intrinsic to the CNSS and may be disrupted by descending pathways. Five out of nine SW-adapted and seven out of eight FW-adapted fish responded in this way to cutting the cord. Electrical stimulation of branchial nerve branches was intended to simulate input to the brain stem from the branchial arches, where osmoreceptors might be expected to be located on the gills. The subsequent inhibition of Dahlgren cell activity further supports the idea of net descending inhibition to the CNSS, which was followed by recovery to the original activity level. Tactile stimulation of lips and fin delivers input to the brain stem *via* the trigeminal nerve, and produced a similar response to branchial nerve stimulation, as well as a possible rebound effect. These findings provide preliminary evidence for descending modulation of the CNSS in response to activation of sensory nerves with potential relevance for the maintenance of osmotic homeostasis. It is likely that both electrical and tactile stimulus regimes led to brainstem arousal and hence descending inhibition.

The Dahlgren cells appear to be subject to a balance of inhibitory and excitatory input, both intrinsic and extrinsic to the CNSS. For example, we have already demonstrated profound inhibition *via* adrenergic receptors (Hubbard et al., 1996b) as well both nicotinic and muscarinic cholinergic modulation (Brierley et al., 2003). Similarly, firing patterns in mammalian hypothalamic oxytocin neurons are regulated *via* a range of excitatory (e.g. noradrenergic) and inhibitory (opioid) inputs (Russell et al., 2003). These cells receive input from the brain stem, which mediates vagal and spinal feedback from cervix and uterus during parturition. Such comparisons further emphasise the parallels that may be drawn between hypothalamic and CNSS magnocellular neuroendocrine systems.

The physiological role of changes in Dahlgren cell firing patterns, in terms of neuroendocrine peptide secretion and target tissue action, remains to be established. However, our earlier observations of altered circulating and urophysial peptide levels (Arnold-Reed et al., 1991; Bond et al., 2002), and their reported actions on gill (Marshall and Bern, 1979), bladder (Loretz and Bern, 1981) and gut (Loretz et al., 1985) transport, are consistent with a predominant function of urotensins to support fish survival in a hypertonic SW environment. The electrophysiological data reported here are in agreement with this picture in so far as SW fish showed higher proportions of bursting cells, with a more robust bursting pattern, and an apparently greater correlation between firing activities of Dahlgren cells in SW compared with FW CNSS. The sensitivity of Dahlgren cell firing patterns to higher centre mediated responses to external sensory input is likely to be of importance, not only for sensing potential challenges of variation in water salinity, but also in enabling a role for the CNSS in modulating cortisol responses to specific stressors (Winter et al., 2000).

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