Electrical activity of caudal neurosecretory neurons in seawater- and freshwater-adapted flounder: responses to cholinergic agonists

M. J. Brierley, A. J. Ashworth, T. P. Craven, M. Woodburn, J. R. Banks, W. Lu, D. Riccardi, R. J. Balment and C. R. McCrohan*

School of Biological Sciences, University of Manchester, Manchester M13 9PT, UK

*Author for correspondence (e-mail: Cathy.McCrohan@man.ac.uk)

Summary

The caudal neurosecretory system (CNSS) of the euryhaline flounder is involved in osmoregulatory responses underlying adaptation to seawater and freshwater. This study compared electrophysiological activity and responses to cholinergic agonists in the neuroendocrine Dahlgren cells in an *in vitro* preparation taken from fully seawater- (SWA) or freshwater-adapted (FWA) fish. Resting membrane and action potential parameters showed few differences between SWA and FWA cells. The hyperpolarisation-activated sag potential and depolarising afterpotential were present under both conditions; however, amplitude of the latter was significantly greater in SWA cells. The proportions of cells within the population exhibiting different firing patterns were similar in both adaptation states. However, bursting parameters were more variable in FWA cells, suggesting that bursting activity was less robust. The muscarinic agonist, oxotremorine, was largely inhibitory in Dahlgren cells, but increased activity in a non-Dahlgren cell population, α neurons. Nicotine promoted bursting activity in SWA Dahlgren cells, whereas it inhibited over half of FWA cells.

Key words: flounder, *Platichthys flesus*, neurosecretory system, Dahlgren cell, acetylcholine, nicotine, oxotremorine, electrophysiology, osmoregulation.

Introduction

The caudal neurosecretory system (CNSS) of teleost fish is located in the terminal vertebral segments of the spinal cord and comprises magnocellular neuroendocrine Dahlgren cells together with a discrete neurohaemal release organ, the urophysis (Winter et al., 2000). There is evidence to support a role for the major neuropeptide secretory products of the CNSS, urotensins I and II (UI and UII), in osmoregulatory responses to altered external salinity and internal osmotic conditions (Bern et al., 1985; Lederis et al., 1985; Minniti and Minniti, 1995; Winter et al., 2000). For the euryhaline flounder *Platichthys flesus*, this includes full adaptation to a wide range of water salinities encountered during their circannual migration between the open sea (ca. 1000 mOsmol l^{-1}) during autumn/winter months and tidal estuarine and river water (as low as 1 mOsmol l^{-1}) in the spring and summer.

Using intracellular recording from an isolated *in vitro* CNSS preparation, Hubbard et al. (1996a) identified two types of Dahlgren cell in the flounder, based on electrophysiological criteria. Type 1 cells were spontaneously active, whereas type 2 cells were less numerous, normally quiescent and relatively inexcitable. The two cell types have similar morphology (Hubbard et al., 1996a) and apparently both colocalise UI and UII (A. Ashworth, unpublished observation; Yamada et al., 1986; Larson et al., 1987; Ichikawa et al., 1988). This raised

the possibility that type 1 and type 2 cells represent different functional states of a common neuron type. Around 60% of type 1 cells exhibit characteristic bursting activity (Brierley et al., 2001), reminiscent of that described for other magnocellular neuroendocrine cells, such as mammalian vasopressin neurons (Armstrong et al., 1994; Stern and Armstrong, 1995; Leng et al., 1999). Bursting activity has been reported to facilitate release of neuropeptide (Cazalis et al., 1985), suggesting that the pattern of electrical activity in these cells may be at least as important as overall firing frequency in regulating release of neurohormone into the circulation.

Using a homologous radioimmunoassay for flounder UII, Winter et al. (1999) reported elevated levels of the peptide in plasma taken from fully seawater-adapted (SWA) compared to freshwater-adapted (FWA) fish. This suggests that the activity patterns of Dahlgren cells may differ between the two states of adaptation, leading to raised UII secretion in seawater conditions. Such differences have not yet been investigated; previous electrophysiological studies in the flounder were confined to CNSS from SWA fish.

Our electrophysiological studies of flounder CNSS *in vitro* have identified neuromodulators of Dahlgren cell activity, which probably arise *in vivo* from descending pathways. For example, superfusion with adrenergic agonists led to

hyperpolarisation of type 1 and type 2 cells (Hubbard et al., 1996b); type 1, but not type 2, cells were similarly inhibited by serotonin (Hubbard et al., 1997). A further potential modulator of CNSS activity is acetylcholine (ACh). Pandey (1981) reported the presence of acetylcholinesterase in CNSS from a number of freshwater teleost species and, in a study using H^{3+} choline uptake, Conlon and Balment (1996) confirmed ACh synthesis within isolated trout CNSS, together with marked ACh release in response to a depolarising (high K^+) stimulus. A preliminary electrophysiological study (Brierley et al., 2000) demonstrated cholinergic excitation of a sub-population of flounder type 1 Dahlgren cells; indeed, ACh is the only neurotransmitter so far shown to depolarise these neurons. This raises the possibility that ACh may play a role in regulating Dahlgren cell activity during the transition between freshwater and seawater.

In this study we first aimed to compare electrophysiological properties and spontaneous activity patterns of Dahlgren cells in CNSS taken from fully SWA and FWA flounders. We then tested the hypothesis that ACh modulates Dahlgren cell activity and that its modulatory effects are related to the adaptive state of the system. Both nicotinic and muscarinic effects were investigated, since mammalian vasopressin neurons are modulated by both these pathways (Michels et al., 1991; Renaud and Borque, 1991; Mori et al., 1994; Zaninetti et al., 2002). We used both intracellular and extracellular (multi-unit) recordings from the isolated CNSS preparation.

Materials and methods

Adult flounder *Platichthys flesus* L. (300–800 g) were caught in Morecambe Bay (Ulverston channel) and housed under a 12 h:12 h light:dark cycle in seawater (SW) (860–1000 mOsmol l^{-1}) or freshwater (FW) (0–6 mOsmol l^{-1}) at 8–12°C. Fish were kept in either SW or FW for a minimum of 14 days to allow full osmotic adaptation before experimentation. Fish were killed using a standard protocol detailed under UK licence procedures (Home Office Schedule 1). The caudal 8–9 segments of the spinal cord and attached urophysis were dissected out and the outer connective sheath removed with fine forceps. The CNSS was placed in a cooled (8–12°C) interface recording chamber and continuously superfused at 1 ml min^{-1} with aerated flounder Ringer (composition in mmol l^{-1}; for SW: K_2HPO_4 1.0, KCl 0.5, NaCl 160, $CaCl_2$ 2.12, $MgSO_4$ 1.0, D-glucose 5.56, Hepes 10, pH 7.7; for FW: K_2HPO_4 1.0, KCl 0.5, NaCl 134, $CaCl_2$ 2.12, $MgSO_4$ 1.0, D-glucose 5.56, Hepes 10, pH 7.7); these compositions represent the osmolality and ionic composition measured in plasma from SWA and FWA fish (Bond et al., 2002). The preparation was allowed to equilibrate for 30–60 min before recording.

Intracellular recordings from Dahlgren cells

Intracellular recordings were made from individual Dahlgren cells using glass microelectrodes (40–80 MΩ when filled with 3 mol l^{-1} potassium acetate). Electrodes were fabricated using a one-stage pull on a Flaming–Brown type P-97 micropipette puller (Sutter Instrument Co., CA, USA) and thin-walled, filamented, borosilicate glass (GC150F-10, Harvard Apparatus, Kent, UK). The electrode was connected to an Axoclamp 2A amplifier (Axon Instruments, CA, USA), data captured (sampling rate ≥8 kHz) *via* a CED 1401 interface (Cambridge Electronic Design, UK) and stored and analysed (off-line) using CED Spike2 (v4.01) software. Once a neuron had been penetrated, the electrode was bridge-balanced (using 500 ms, –0.3 nA pulses at 0.3 Hz) and the cell's spontaneous electrical activity and resting membrane potential were monitored for at least 30 min. Cells with membrane potentials more negative than –50 mV and that generated the action potential waveform characteristic of Dahlgren cells (Hubbard et al., 1996a) were considered viable. Stable recordings of up to 3 h were possible. Measurement protocols for membrane parameters followed Hubbard et al. (1996a). For measurement of action potential duration (measured at half maximal height) only action potentials which were not preceded by another spike for >10 s were sampled, as Dahlgren cells exhibit frequency-dependent spike broadening (Brierley et al., 2001).

Extracellular recordings from the CNSS

Extracellular recordings were made from pre-terminal segments 2–4 using a large (300–600 µm tip diameter) glass suction electrode, filled with Ringer. The electrode was placed directly on the surface of the cord to record activity in nearby neuronal somata and axonal tracts. Signals were recorded differentially and amplified (×10K) using a Neurolog AC NL104 amplifier (Digitimer, UK) and filtered (AC NL125; 5 Hz and 1.2 kHz cut-off frequencies plus a 50 Hz notch filter). A CED converter and Spike2 (v4.01) software were used for data storage (sampling rate 8–12 kHz) and analysis. Stable recordings of up to 8 h were possible. Recordings were analysed off-line using CED Spike2 software to separate out activity of up to eight individual units. Briefly, the software generates an action potential template for each unit and scans the recording for similar waveforms (60–90% fit required). Activity from Dahlgren cells was positively identified by their long duration (4–8 ms), triphasic action potential waveform. Units were selected randomly but only included in the final analysis when >90% (estimated empirically) of activity was successfully extracted from the original recording. Action potentials from separated units are illustrated as event marks using the Spike2 software option. Estimation of overall activity during extracellular recordings was achieved using the channel process option, in which waveforms are rectified (all negative values replaced by positive values) and then integrated *via* the smooth function (time constant=1 s).

Pharmacological experiments

Drugs (ACh, Sigma-Aldrich, UK; nicotine, RBI, UK; oxotremorine M, Tocris Cookson, UK) (100 µmol l^{-1} in appropriate Ringer) were cooled to 8–12°C prior to bath superfusion over the CNSS. Drugs were superfused for 10 min,

Table 1. *Membrane and action potential parameters of type 1 and type 2 Dahlgren cells from seawater- and freshwater-adapted caudal neurosecretory systems*

	Type 1 Dahlgren cells		Type 2 Dahlgren cells	
	SWA	FWA	SWA	FWA
Membrane parameters				
Resting membrane potential (mV)	–61.4±1.5 (25)	–60.8±1.5 (18)	–60.3±1.6 (7)	–62.3±2.8 (3)
Input resistance (MΩ)	25.6±1.9 (12)	32.1±3.9 (15)	24.1±1.6 (7)	21.1±3.1 (3)
Action potential parameters				
Threshold (mV)	–49.7±1.8 (12)	–48.2±1.3 (18)	–44.2±1.1 (7)	–45.9±0.5 (3)
Amplitude (mV)	89.9±2.0 (21)	92.0±1.8 (18)	68.6±2.7 (7)[††]	75.0±1.0 (3)[†††]
Duration (ms)	4.3±0.1 (9)	3.5±0.2 (6)*	4.5±0.2 (7)	3.4±0.1 (3)*
Afterhyperpolarisation amplitude (mV)	11.9±0.9 (21)	12.7±0.5 (18	7.5±0.6 (7)[†]	8.7±0. 6 (3)[†††]
Afterhyperpolarisation duration (ms)	209.8±20.0 (21)	217.6±19.2 (18)	36.6±2.5 (7)[††]	32.0±2.5 (3)[†††]

SWA, seawater adapted; FWA, freshwater adapted.
Values are means ± S.E.M. (number of cells).
Only spike duration was significantly different between SWA and FWA cells.
*Significant differences between type 1 or type 2 cells from SWA *versus* FWA preparations; *$P<0.02$.
[†]Significant differences between type 1 and type 2 cells from the same adaptation state; [†]$P<0.02$, [††]$P=0.01$, [†††]$P<0.01$.

followed by a return to normal Ringer. There was a minimum of 20 min between successive drug applications.

Data analysis

Data are presented as mean ± S.E.M., except for burst data (Table 2), which are presented as ± S.D. to allow assessment of the variability in durations of different phases of activity. Statistical analysis (student's two tailed *t*-test) was carried out using Graphpad Prism (v3.0).

Results

Intracellular recording from Dahlgren cells

Table 1 presents resting membrane and action potential parameters of type 1 and type 2 Dahlgren cells from SWA and FWA fish. For SWA cells, these data agree with those reported by Hubbard et al. (1996a); action potential amplitude was

significantly greater in type 1 compared to type 2 cells, as were amplitude and duration of spike afterhyperpolarisation (AHP). In particular, AHP duration was about 5 times greater in type 1 compared to type 2 cells (Table 1, Fig. 1Ai). Similar differences were found between the two cell types from FWA preparations (Table 1, Fig. 1Aii). There were no differences in membrane and action potential parameters of the same cell type from SWA compared to FWA CNSS, with one exception. Action potential duration in type 1 and type 2 cells (measured at half maximal amplitude) was significantly longer in SWA compared to FWA preparations (Table 1).

Type 1 cells from SWA CNSS exhibit a 'sag' potential in response to a hyperpolarising injected current pulse, which is more pronounced at depolarised membrane potentials (Brierley et al., 2001). In this study, we found similar sag potentials in type 1 cells from FWA CNSS (Fig. 1). Furthermore, as for SWA cells (Brierley et al., 2001), the sag potential in FWA

Fig. 1. Responses to depolarising and hyperpolarising current pulses in type 1 and type 2 Dahlgren cells recorded intracellularly from SWA and FWA preparations. (Ai,ii) Both SWA (i) and FWA (ii) cells fire in response to a depolarising current pulse (+0.9 nA, 300 ms) and on the rebound following a hyperpolarising (–0.9 nA, 300 ms) pulse. (B) Voltage-dependent sag potentials (dotted lines) and depolarizing afterpotentials (DAP, arrow) recorded from a type 1 FWA cell in response to hyperpolarising square-wave current injection (–1.2 nA, 300 ms). The membrane potential was held at three levels [–50, –60 (RMP) and –70 mV] by constant current injection. At the more depolarised potential, the DAP triggered an action potential. RMP, resting membrane potential.
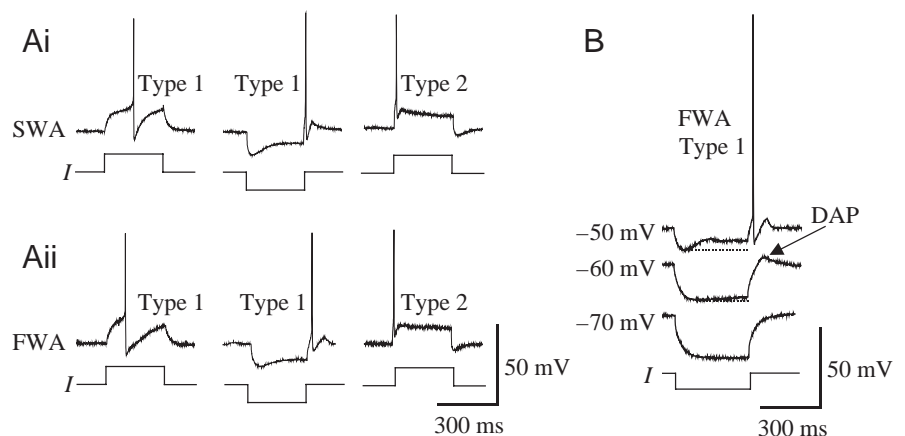
cells was voltage-dependent. This is illustrated in Fig. 1B, which shows responses to an injected hyperpolarising current pulse (–1.2 nA) in a FWA type 1 Dahlgren cell, measured at three levels of membrane potential (resting membrane potential, RMP=–60 mV, and RMP±10 mV). The degree of sag (measured as the difference in membrane potential between the initial peak deflection and that at pulse termination) for RMP+10 mV (6.0±2.1 mV) was significantly greater (*P*<0.05, *N*=10 cells) than that measured at RMP (1.7±0.4 mV) and RMP–10 mV (0.4±0.2 mV). There was no significant difference between sag potentials in FWA compared to SWA cells at RMP or at RMP±10 mV.

The sag potential is followed by a depolarising after-potential (DAP) immediately following termination of the

current pulse in both SWA (Fig. 1A; Brierley et al., 2001) and FWA (Fig. 1) cells. Like the sag potential, this DAP is voltage-dependent. At RMP and more negative potentials, DAP amplitude was <2 mV and not significantly different between SWA (*N*=10) and FWA (*N*=6) cells (*P*>0.5). However, at the depolarised membrane potential (RMP+10 mV) DAP amplitude was significantly greater (*P*<0.005) than at more negative potentials, sometimes causing the cell to overshoot threshold and fire (Fig. 1B). In addition, DAPs evoked in depolarised SWA type 1 cells were significantly larger than in depolarised FWA type 1 cells (SWA=7.0±0.7 mV, *N*=10; FWA=3.6±0.5 mV, *N*=6; *P*=0.005).

Recordings from type 2 cells (SWA 8 cells; FWA 3 cells; not shown) revealed the presence of both sag potentials and DAP, suggesting similar membrane properties in spontaneously active type 1 and in the relatively inexcitable type 2 Dahlgren cells. Sag potentials (ca. 1 mV at RMP) and DAPs (ca. 2.5 mV) were not significantly different between SWA and FWA type 2 cells.

Since type 2 cells are not spontaneously active, and therefore not recorded extracellularly, the remaining analyses described in this paper were confined to type 1 Dahlgren cells.

Extracellular recording from CNSS

In some preparations (*N*=8) a type 1 Dahlgren cell was recorded simultaneously both intracellularly, from the more rostral soma, and extracellularly from the descending axon tract. This allowed measurement of axonal conduction velocity (0.73±0.16 ms^{-1}), which indicates, as suggested by previous histochemical evidence (Pandey, 1981), that the Dahlgren cell axons are unmyelinated. Extracellular recording revealed typical long-duration action potentials (4–8 ms when recorded extracellularly) characteristic of Dahlgren cells (Fig. 2A). It also revealed the presence of electrical activity from another, unidentified, neuron type with much shorter duration action potentials (<1 ms), indicating the presence of non-Dahlgren cells (here termed α neurons; Fig. 2A) within the CNSS. Spontaneous α neuron activity was more commonly recorded from FWA (ca. 50% of recordings) than from SWA (ca. 20% of recordings) preparations but was always triggered following muscarinic activation (see below).

Suction electrode recording allowed us to monitor the activity of several Dahlgren cells in the same preparation. Fig. 2B shows an example of a SWA recording analysed using Spike2 to separate out activity of individual units. Typically between 2 and 6 units were analysed in this way in each preparation. Cells from SWA (*N*=40 from 8 fish) and FWA (*N*=40 from 12 fish) preparations were recorded for 60 min. Activity was classified as: 'bursting', comprising discrete action potential bursts of >20 s separated by periods of >20 s inactivity; 'phasic', consisting of irregular activity including periods of higher frequency (typically 1–3 Hz), lower frequency (<1 Hz) and no firing; 'tonic' with continuous activity of ca. 1 Hz and few or no silent periods; 'silent', cells whose presence only became
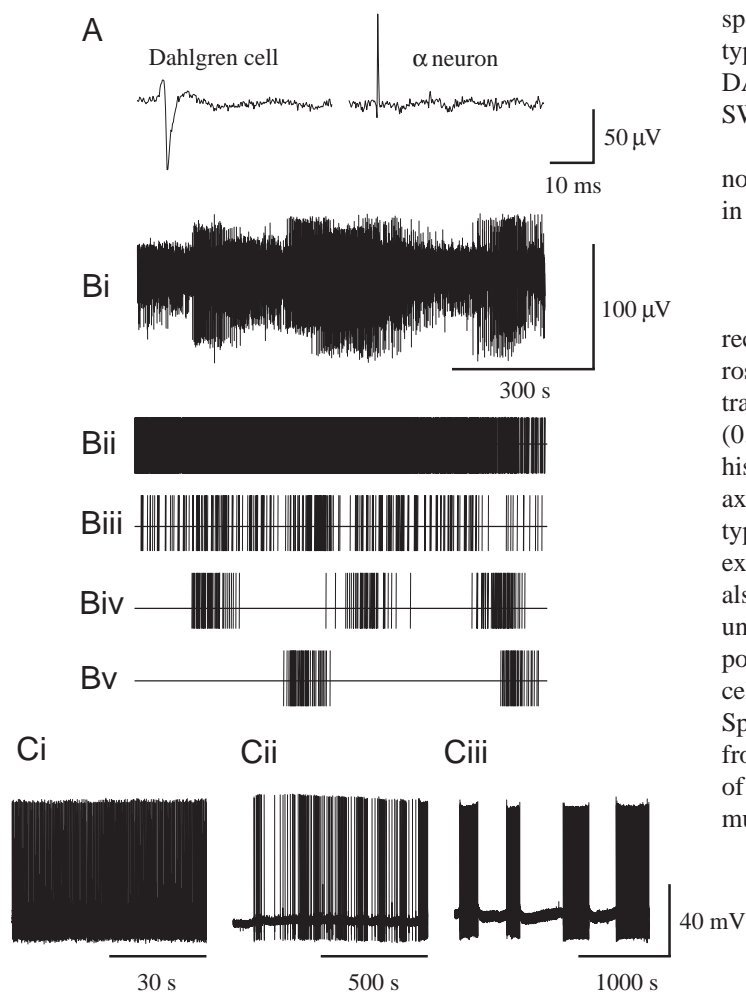

Fig. 2. (A) Extracellular recordings of action potentials from CNSS. Dahlgren cell shows a triphasic, long-duration (ca. 8 ms) action potential waveform. An unidentified cell type, α neuron, generates a short duration (<1 ms) action potential. (B) Multiunit extracellular recording (i) of spontaneously firing Dahlgren cells in SWA CNSS, analysed off-line using Spike2 to distinguish activity (action potentials displayed by the software as event marks) in four separate units (ii–v). Three different firing patterns are seen: tonic (ii), phasic (iii) and bursting activity (iv,v). (C) Intracellular recordings from three type 1 Dahlgren cells, illustrating spontaneous tonic (i), phasic (ii) and bursting activity patterns (iii).

apparent when the cell became spontaneously active during the recording period. Similar activity patterns were also distinguished from intracellular recordings (Fig. 2C). Table 2 summarises the firing activity of bursting, phasic and tonically active Dahlgren cells from SWA and FWA CNSS (silent cells could not be included in this analysis). The proportions of cells displaying different classes of activity pattern did not differ between SWA and FWA preparations. Furthermore, there was no significant difference in firing frequencies (mean or maximum), or of burst duration or cycle period in bursting cells. However, FWA bursting cells were more variable in terms of burst duration and cycle period compared to SWA, as shown by the larger S.D. for these parameters for FWA cells. This greater variation was mainly accounted for by a longer deceleration phase for FWA bursts. The minimum burst duration from FWA and SWA cells was similar (34 s and 42 s respectively), as was the mean acceleration phase duration (i.e. time from the onset of a burst to peak spike frequency). However, although the mean deceleration phase duration was not significantly different ($P>0.5$), the coefficients of variation for this parameter were very different at 49% for SWA and 91% for FWA, suggesting a difference in the mechanisms underlying SWA and FWA burst termination.

Transitions between firing patterns in individual Dahlgren cells

During the 60 min extracellular recording period, some units underwent a spontaneous transition between one type of firing pattern and another (Fig. 3), supporting our view that different classes of activity do not represent different classes of Dahlgren cell, but rather that the same cell type may fire in different ways.

Spontaneous transitions occurred in 7/40 (17.5%) SWA and 11/40 (27.5%) FWA Dahlgren cells. All of the transitions in SWA Dahlgren cells involved tonic activity. The majority were between tonic activity and silence (5/7 cells: 71%; Fig. 3A) and two (29%) involved tonic–phasic transitions. There were no transitions to or from bursting activity in SWA Dahlgren cells. FWA cells underwent a greater variety of transitions (Fig. 3B). Tonically active FWA cells underwent transitions to all other firing patterns (one became bursting, one phasic and two silent) and two silent cells became active (one tonic, one bursting). In

contrast to the SWA observations, 36% of the transitions involved a change to bursting activity (Fig. 3B) and there was a single observation of a regularly bursting neuron becoming phasic.

Responses to acetylcholine and ACh receptor agonists

In a preliminary study, Brierley et al. (2000) observed both depolarising and hyperpolarising responses to acetylcholine in Dahlgren cells and, in a subset of cells, no response at all. In this study, superfusion with ACh (100 µmol l⁻¹) elicited similar inconsistent responses in cells recorded extracellularly,

Table 2. *Spontaneous activity patterns in seawater- and freshwater-adapted type 1 Dahlgren cells (extracellular recordings)*

Type of neurons	Dahlgren cells	
	SWA	FWA
Tonically active		
% of total	20	15
Mean spike frequency (Hz)	0.84±0.61	0.99±0.64
Phasically active		
% of total	20	20
Mean spike frequency (Hz)	0.27±0.2	0.11±0.1
Maximum spike frequency (Hz)	1.56±0.68	3.7±1.41
Bursting		
% of total	60	65
Cycle period (s)	747.10±277.6	831.0±467.3
Burst duration (s)	131.90±69.3	181.40±255.1
Mean spike frequency (per 60 min) (Hz)	0.13±0.1	2.10±1.3
Maximum frequency/burst (Hz)	1.82±1.17	1.31±0.4
Mean frequency/burst (Hz)	1.15±0.7	1.14±0.5
Burst acceleration phase duration (s)	57.70±33.9	54.50±39.5
Burst deceleration phase duration (s)	98.40±48.4	122.10±110.6

SWA, seawater adapted; FWA, freshwater adapted.

Values are means ± S.D.; $N=40$ for both adaptation states.

Parameters were similar for SWA and FWA neurons, but burst activity in FWA cells exhibited greater variability.
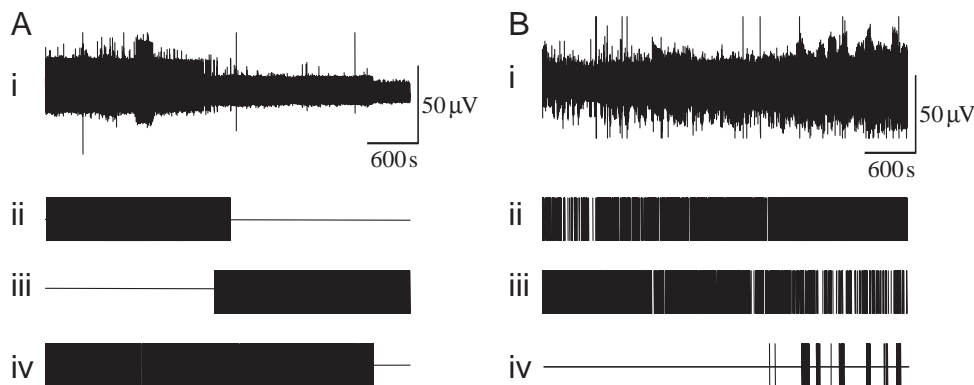

Fig. 3. Spontaneous transitions between different activity patterns in single units identified from multiunit recordings from CNSS. (A) SWA recording (i) in which two tonically active Dahlgren cells become silent (ii,iv) and a quiescent cell becomes tonically active (iii). (B) FWA recording (i) in which one cell switches spontaneously from phasic to tonic activity (ii), one from tonic to phasic activity (iii) and a third, quiescent, cell becomes bursting (iv).
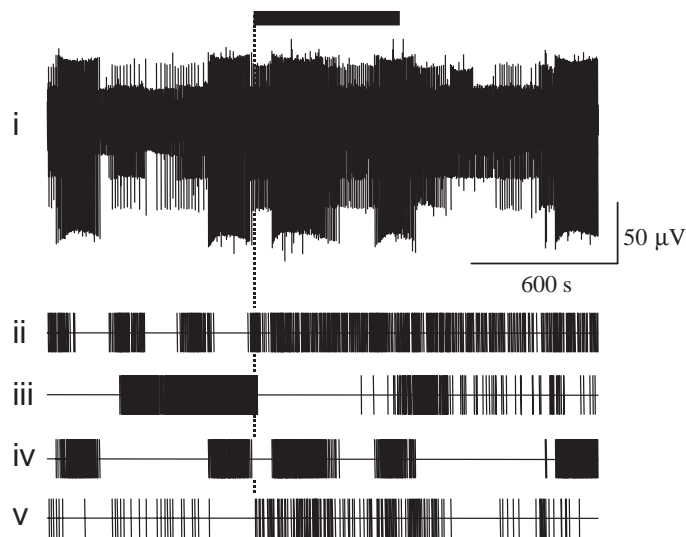
Fig. 4. Responses of Dahlgren cells to superfusion with acetylcholine (100 μmol l^{-1}, 600 s; solid horizontal bar, onset shown by dotted line) in a SWA CNSS preparation. The activity of four units (ii–v) is separated from the original multiunit recording (i) and shows a range of responses, including a change from bursting to phasic activity (ii), short-term inhibition (iii), and increased firing frequency in a phasically active cell (v).

from SWA (N=3 preparations) (Fig. 4) and FWA (N=3) CNSS. In the example shown in Fig. 4 cells were excited (Fig. 4v), inhibited (Fig. 4iii) and/or altered their firing pattern (Fig. 4ii, bursting to phasic). This range of responses suggested differential ACh receptor (AChR) expression in individual neurons. The contribution of ionotropic and metabotropic AChR was assessed using the agonists nicotine and oxotremorine, respectively.

In both SWA and FWA preparations, superfusion of the broad-spectrum muscarinic agonist, oxotremorine (100 μmol l^{-1}, 600 s) led to an increase in overall activity in the CNSS. However, this was largely due to excitation of α neurons, seen as a marked increase in frequency of short duration (<1 ms) action potentials (Fig. 5A). The effects of oxotremorine on α neurons occurred within 30 s of onset of superfusion and typically washed out within 200 s. Extracellular recording sites varied in their access to Dahlgren cell and α neurons. Some recordings had little or no Dahlgren cell activity (Fig. 5A), whereas others contained activity from >4 Dahlgren cells (Fig. 5B,C). In the latter, α neuron activity often became apparent only after further analysis (e.g. Fig. 5B) as the small-amplitude α neuron signal (<20 μV) was obscured. However, rectification and integration (RIT) of the raw data signal to enable quantification of total activity, highlighted underlying excitation by oxotremorine (Fig. 5B).

The effect of oxotremorine on Dahlgren cells was largely inhibitory, leading to cessation of activity followed by recovery after 10–20 min washout (Figs 5B,C, 6A,B). All FWA type 1 cells (18 units from 7 fish) and 93% of SWA cells
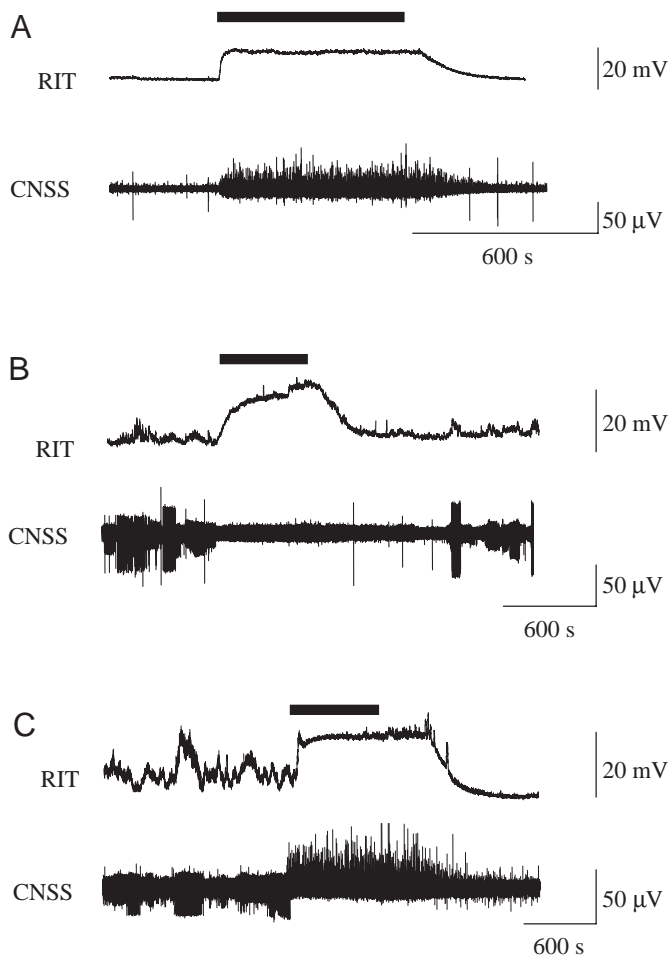

Fig. 5. Superfusion of SWA CNSS with oxotremorine (100 μmol l^{-1}, 600 s; solid horizontal bars) increased activity of α neurons but inhibited Dahlgren cell activity, recorded extracellularly. Upward deflections in rectified integrated (RIT) traces reflect an increase in total activity in the CNSS. (A) No spontaneous Dahlgren cell or α neuron activity is recorded; oxotremorine activated α neurons, as shown in both RIT and voltage (CNSS) traces. (B,C) Several spontaneously active Dahlgren cells are inhibited by oxotremorine. However, an upward deflection in the RIT trace reflects an increase in overall activity due to α neuron spikes.

(26/28 cells from 9 fish) ceased firing following the onset of superfusion (Fig. 6A,B). Following washout with normal Ringer, 68% of SWA and 33% of FWA cells resumed their previous activity pattern. The remaining cells generated a new pattern of activity or remained silent.

The inhibitory effect of oxotremorine was examined further using intracellular recordings. In FWA type 1 Dahlgren cells, oxotremorine induced hyperpolarisation in 4/6 cells (three tonically and one phasically active) (Fig. 6C) leading to cessation of activity for up to 1200 s. Peak hyperpolarisation (20.5±5.2 mV) was reached within 200 s, after which, even in the continued presence of the agonist, membrane potential began to repolarise towards RMP (Fig. 6C). The two non-inhibited neurons (one silent, one bursting) were both
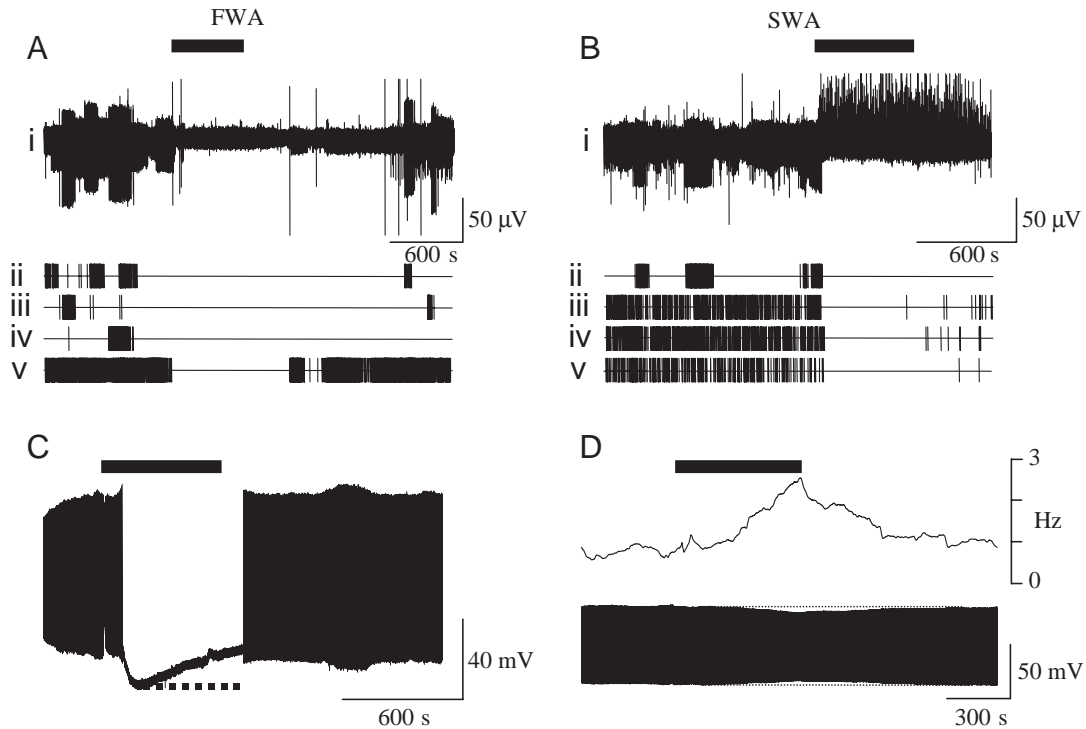
Fig. 6. Effects of oxotremorine (100 µmol l⁻¹, 600 s; horizontal bars) on FWA and SWA Dahlgren cells. (A,B) Activity of individual Dahlgren cells (ii–v) extracted from multiunit recordings (i) from FWA (A) and SWA (B; same recording as Fig. 5C) CNSS. In both recordings, bursting and phasically active Dahlgren cells ceased firing, at least for the duration of the superfusion period. (C) Intracellular recording of FWA type 1 Dahlgren cell, showing hyperpolarisation of membrane potential (ca. 20 mV) in response to oxotremorine. Repolarisation (broken line) occurred in the continuing presence of the agonist. (D) Instantaneous firing frequency (top), and voltage recording (bottom) from a single, tonically active, SWA Dahlgren cell, showing a slow onset increase in firing frequency (from 1 to 3 Hz) in response to oxotremorine, accompanied by a reduction in spike amplitude (between horizontal dotted lines).

transiently and weakly depolarized, by around 5 mV, during the superfusion period only. Three SWA type 1 Dahlgren cells, recorded intracellularly (two tonically active and one bursting) were superfused with oxotremorine. One of the tonically active neurons showed no response, the second was depolarized (Fig. 6D), eliciting an increase (from 1 to 3 Hz) in firing rate. The bursting neuron was transiently hyperpolarized, by about 20 mV, followed by membrane repolarization as described above for FWA cells.

Superfusion with nicotine (100 µmol l⁻¹, 600 s) induced bursting activity in previously non-bursting SWA Dahlgren cells (*N*=8/18 (44%) non-bursting cells) recorded intra- (Fig. 7A) or extracellularly (not shown). However, nicotine had no effect on SWA neurons showing ongoing burst patterns (*N*=11 spontaneously bursting cells; Fig. 7B). A much smaller proportion of non-bursting FWA Dahlgren cells responded to nicotine in this way, with only 3/21 cells (14%) cells generating burst patterns. For both SWA and FWA cells, the transition to bursting typically took >300 s to occur after the initial onset of nicotine superfusion.

No SWA Dahlgren cells were inhibited by nicotine, but 57% (13/23 cells) of FWA cells were. This is illustrated in Fig. 7C, in which three FWA bursting cells were inhibited during or following superfusion with nicotine. Nicotine did not evoke α

neuron activity (not shown) in either SWA or FWA CNSS (data from 10 fish).

Discussion

The membrane and action potential parameters, as well as firing and bursting patterns, of SWA Dahlgren cells reported here compare well with those described earlier (Hubbard et al., 1996a; Brierley et al., 2001). Perhaps surprisingly, recordings of the same parameters from FWA cells showed few differences. Resting membrane and action potential parameters were largely the same, suggesting that there are few, if any, intrinsic differences between Dahlgren cells under different osmotic adaptation states. Spike width was greater in somata of types 1 and 2 cells from SWA compared to FWA fish. If this is also true at the terminals, it could lead to increased neurohormone release per action potential. Furthermore, the potential for action potential broadening during each burst (Brierley et al., 2001), possibly leading to enhanced neuropeptide release as proposed by Cazalis et al. (1985) for oxytocin neurons, might be greater in SWA cells with their intrinsically longer duration action potentials.

The sag potential and depolarising after-potential (DAP) have been suggested as a mechanism to facilitate repetitive
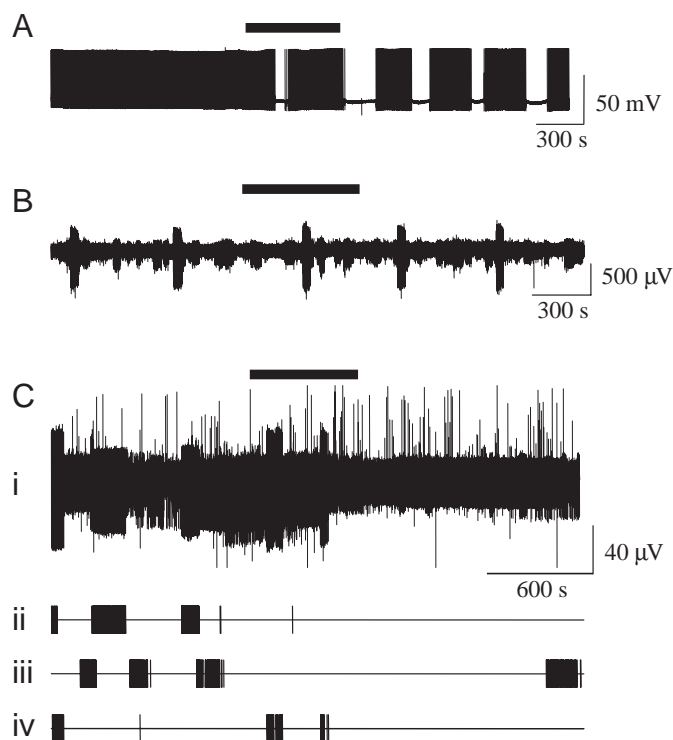
Fig. 7. (A,B) Superfusion with nicotine (100 μmol l⁻¹, 600 s; horizontal bars) promotes bursting activity in SWA Dahlgren cells. (A) Nicotine induces a transition from tonic to bursting activity in a Dahlgren cell recorded intracellularly. (B) Extracellular recording shows no change in ongoing bursting activity in at least three Dahlgren cells in response to nicotine. (C) Superfusion with nicotine (100 μmol l⁻¹, 600 s; horizontal bar) inhibits Dahlgren cells in FWA CNSS. Three bursting units extracted from an extracellular recording become silent following application of nicotine; only one resumes firing after 15 min washout.

firing (bursting) in Dahlgren cells (Brierley et al., 2001). DAPs occur following the action potential afterhyperpolarisation (AHP), taking the membrane potential towards threshold for the next spike. Although the ion channel activation underlying this response has yet to be characterised in these cells, it is reminiscent of the excitatory hyperpolarisation-activated cation current (I_H) described for other rhythmic neuronal and cardiac pacemaker cells (Irisawa, 1987; Erickson et al., 1993; Vasilyev and Barish, 2002). Voltage-dependent sag potentials during hyperpolarising current injection were observed in FWA as well as SWA cells, especially when held at depolarised membrane potentials. However, the DAP was significantly larger in SWA compared to FWA cells, which might be expected to lead to more effective maintenance of ongoing firing activity in the former. However, this was not reflected in a difference in burst parameters between SWA and FWA cells. Although type 2 cells are usually relatively inexcitable, they do appear to generate a sag potential response, reinforcing the hypothesis that type 2 and type 1 Dahlgren cells may represent different activity states within a single neuronal population.

Not only were similar patterns of activity recorded from SWA and FWA Dahlgren cells, but also the proportions of cells showing these patterns of activity were not different. This was unexpected bearing in mind the known involvement of the CNSS and the urotensins in osmoregulatory adaptation (for a review, see Winter et al., 2000). There are several possible explanations for this. The first is the absence of extrinsic input to the CNSS in the *in vitro*, isolated preparation used here. Studies using a range of species have identified descending neuromodulatory input to the CNSS, including monoaminergic pathways (Audet and Chevalier, 1981; Miller and Kriebel, 1986; McKeon et al., 1988; Yulis et al., 1990; Oka et al., 1997; Hubbard et al., 1996b, 1997). It is possible that differences in Dahlgren cell activity following SW–FW adaptation depend on ongoing descending input and are thus not retained *in vitro*. Secondly, any link between electrical activity of the neuroendocrine cells and neuropeptide release from their terminals is unlikely to be straightforward. The amount of peptide released from the storage organ (urophysis) in response to depolarisation may depend more on the level of peptide available for release; we previously showed that the amount of stored peptide in the urophysis is significantly greater in fully SWA compared to FWA flounder (Winter et al., 1999). Local control of release at the level of the terminals is also a possibility; this would not necessarily be reflected by changes in Dahlgren cell activity. However, this raises the question of the function of patterned, and especially bursting, activity in these cells.

Since Dahlgren cells were often seen to change their activity pattern from one type to another ('transitions'), it is proposed that they represent a fairly homogeneous population in which the activity of individuals can change over time. It was assumed that extracellular recordings included only type 1 neurons, since type 2 are quiescent, relatively inexcitable and show pronounced spike frequency accommodation when induced to fire (Hubbard et al., 1996a). However, type 1 neurons too may often be silent (16% of SWA cells; Brierley et al., 2001). It was not possible using extracellular recording to quantify the proportion of silent type 1 cells in SWA and FWA preparations so there remains the possibility that more cells are quiescent in FWA fish, leading to reduced peptide release.

Transitions between activity patterns showed some differences between SWA and FWA preparations. Specifically, spontaneous transitions in SWA Dahlgren cells never involved bursting activity, whereas transitions to or from bursting activity were common in FWA cells. This suggests that bursting activity may be more stable in SW than in FW conditions. Further evidence for this is the greater variability of burst cycle period and duration observed in FWA compared to SWA cells. Differences in the robustness of bursting activity could result from the greater variability in osmoregulatory state of individual fish in FW. Although plasma osmolality is tightly maintained by SWA fish (ca. 324 mOsmol kg⁻¹ H_2O; Bond et al., 2002) osmolality in FWA fish is much more variable, as indicated by significantly

greater S.D. (H. Bond, personal communication), suggesting that individual fish cope with low salinity media with differing degrees of precision.

The effects of ACh and agonists reported here support the hypothesis that ACh plays a role in regulating CNSS activity. Central cholinergic pathways have profound effects on rhythmic neuronal activity in a number of systems. For example, in the mammalian hippocampus (Cobb et al., 1999), supraoptic nucleus (Zaninetti et al., 1999), somatosensory cortex (Buhl et al., 1998) and entorhinal cortex (Klink and Alonso, 1997), ACh can initiate burst-mode activity and promote synchrony within neuronal populations. Furthermore, mammalian osmoregulatory vasopressin neurons, which have comparable activity patterns to bursting Dahlgren cells (Armstrong et al., 1994), are stimulated *via* both nicotinic and muscarinic pathways. The main source of ACh input to the flounder CNSS *in vivo* is unclear. Conlon and Balment (1996) demonstrated ACh synthesis and release in the isolated CNSS. However, descending spinal pathways involving ACh may also be present, as reported for several freshwater teleosts by Pandey (1989).

Muscarinic actions of ACh on both SWA and FWA Dahlgren cells were largely inhibitory. Indeed some cells showed 20 mV hyperpolarisation in response to oxotremorine, which is consistent with the presence of muscarinic receptors on the Dahlgren cells themselves. In addition, oxotremorine caused marked activation of α neurons. The identity of these neurons is unknown. They are unlikely to be motoneurons, as these are not thought to be present in the terminal segments of spinal cord. α neurons showed more spontaneous activity in FWA than in SWA CNSS, suggesting that they may have a role related to the osmoregulatory function of the system. Cohen et al. (1990) described a dense plexus of serotonin immunoreactive fibres in the CNSS of the molly (*Poecilia latipinna*). It is possible that α neurons correspond to these fibres and might therefore mediate at least some of the reported inhibition of Dahlgren cells *via* serotonin (Hubbard et al., 1997).

Acetylcholine appeared to promote bursting activity in Dahlgren cells, *via* nicotinic receptors. However, this effect was more pronounced in SWA than in FWA CNSS. This would be expected to enhance release of urotensins. In FWA CNSS, more than half of cells recorded were inhibited by nicotine. Thus responses of FWA Dahlgren cells, both muscarinic and nicotinic, were largely inhibitory.

Cholinergic effects on Dahlgren cells appear to involve a balance between reducing overall activity and promoting secretion-efficient bursting. In FWA CNSS, responses tend towards inhibition, whereas in SWA fish, bursting activity is promoted. These differential effects of AChR ligands on SWA and FWA preparations, though subtle, may contribute, together with those of other modulators, to differential activity patterns *in vivo*. Our results suggest that differences in functional expression of ACh receptors may occur during SW–FW adaptation, thus altering the Dahlgren cells' response to descending inputs.

References

Armstrong, W. E., Smith, B. N. and Tian, M. (1994). Electrophysiological characteristics of immunochemically identified rat oxytocin and vasopressin neurons *in vitro*. *J. Physiol.* **475**, 115-128.

Audet, C. and Chevalier, G. (1981). Monoaminergic innervation of the caudal neurosecretory system of the brook trout *Salvelinus fontinalis* in relation to osmotic stimulation. *Gen. Comp. Endocrinol.* **45**, 189-203.

Bern, H. A., Pearson, D., Larson, B. A. and Nishioka, R. S. (1985). Neurohormones from fish tails: the caudal neurosecretory system. I. 'Urophysiology' and the caudal neurosecretory system of fishes. *Rec. Progr. Horm. Res.* **41**, 533-552.

Bond, H., Winter, M. J., Warne, J. M., McCrohan, C. R. and Balment, R. J. (2002). Plasma concentrations of arginine vasotocin and urotensin II are reduced following transfer of the euryhaline flounder (*Platichthys flesus*) from seawater to fresh water. *Gen. Comp. Endocrinol.* **125**, 113-120.

Brierley, M. J., McCrohan, C. R. and Balment, R. J. (2000). Cholinergic excitation of the urotensin-containing neurosecretory cells in the spinal cord of the flounder, *Platichthys flesus, in vitro*. *J. Physiol.* **523P**, 274.

Brierley, M. J., Ashworth, A. J., Banks, J. R., Balment, R. J. and McCrohan, C. R. (2001). Bursting properties of caudal neurosecretory cells in the flounder *Platichthys flesus, in vitro*. *J. Exp. Biol.* **204**, 2733-2739.

Buhl, E. H., Tamas, G. and Fisahn, A. (1998). Cholinergic activation and tonic excitation induce persistent gamma oscillations in mouse somatosensory cortex *in vitro*. *J. Physiol.* **513**, 117-126.

Cazalis, M., Dayanithi, G. and Nordmann, J. J. (1985). The role of patterned burst and interburst interval on the excitation-coupling mechanism in the isolated rat neural lobe. *J. Physiol.* **369**, 45-60.

Cobb, S. R., Bulters, D. O., Suchak, S., Riedel, G., Morris, G. M. and Davies, C. H. (1999). Activation of nicotinic acetylcholine receptors patterns network activity in the rodent hippocampus. *J. Physiol.* **518**, 131-140.

Cohen, S. L., Miller, K. E. and Kriebel, R. M. (1990) Distribution of serotonin in the caudal neurosecretory complex: a light and electron microscopic study. *Anat. Embryol.* **181**, 491-498.

Conlon, M. J. and Balment, R. J. (1996). Synthesis and release of acetylcholine by the isolated perifused trout caudal neurosecretory system. *Gen. Comp. Endocrinol.* **103**, 36-40.

Erickson, K. R., Ronnekleiv, O. K. and Kelly, M. J. (1993). Electrophysiology of guinea-pig supraoptic neurones: role of a hyperpolarization-activated cation current in phasic firing. *J. Physiol.* **406**, 407-425.

Hubbard, P. C., Balment, R. J. and McCrohan, C. R. (1996a). Electrophysiological characteristics of cells of the caudal neurosecretory system in the teleost, *Platichthys flesus. Comp. Biochem. Physiol.* **115A**, 293-301.

Hubbard, P. C., Balment, R. J. and McCrohan, C. R. (1996b). Adrenergic receptor activation hyperpolarizes the caudal neurosecretory cells of the flounder *Platichthys flesus. J. Neuroendocrinol.* **8**, 153-159.

Hubbard, P. C., Balment, R. J. and McCrohan, C. R. (1997). Inhibitory effect of 5-hydroxytryptamine receptor activation on caudal neurosecretory cells in the flounder, *Platichthys flesus. J. Neuroendocrinol.* **9**, 561-566.

Ichikawa, T., Ishida, I., Ohsako, S. and Deguchi, T. (1988). *In situ* hybridisation demonstrating coexpression of urotensins I, II-alpha, and II-gamma in the caudal neurosecretory neurons of the carp, *Cyprinus carpio. Gen. Comp. Endocrinol.* **71**, 493-501.

Irisawa, H. (1987). Membrane currents in cardiac pacemaker tissue. *Experientia* **43**, 1131-1135.

Klink, R. and Alonso, A. (1997). Muscarinic modulation of the oscillatory and repetitive firing properties of entorhinal cortex layer II neurons. *J. Neurophysiol.* **77**, 1813-1828.

Larson, B. A., Bern, H. A., Lin, R. J. and Nishioka, R. S. (1987). A double sequential immunofluorescence method demonstrating the co-localisation of urotensins I and II in the caudal neurosecretory system of the teleost, *Gillichthys mirabilis. Cell. Tissue Res.* **247**, 233-239.

Lederis, K., Fryer, J. N. and Yulis, C. R. (1985). The fish neuropeptide urotensin I: its physiology and pharmacology. *Peptides* **6**, 353-361.

Leng, G., Brown, C. H. and Russell, J. A. (1999). Physiological pathways regulating the activity of magnocellular neurosecretory cells. *Prog. Neurobiol.* **57**, 625-655.

McKeon, T. W., Cohen, S. L., Black, E. E., Kriebel, R. M. and Parsons, R. L. (1988). Monoamines in the caudal neurosecretory complex: biochemistry and immunohistochemistry. *Brain Res. Bull.* **21**, 37-42.

Michels, K. M., Meeker, R. B. and Hayward, J. N. (1991). Muscarinic cholinergic control of vasopressin secretion from the acute hypothalamoneurohypophysial explant. *Neuroendocrinol.* **54**, 219-226.

Miller, K. E. and Kriebel, R. M. (1986). Peptidergic innervation of caudal neurosecretory neurons. *Gen. Comp. Endocrinol.* **64**, 396-400.

Minniti, F. and Minniti, G. (1995). Immunocytochemical and ultrastructural changes in the caudal neurosecretory system of a seawater fish *Boops boops L.* (teleostei: sparidae) in relation to the osmotic stress. *Eur. J. Morphol.* **33**, 473-483.

Mori, M., Tsushima, H., Kamiya, T. and Matsuda, T. (1994). Effect of muscarinic receptor modulators in the hypothalamic supraoptic nucleus of the rat. *Jpn J. Pharmacol.* **66**, 413-419.

Oka, S., Chiba, A. and Honma, Y. (1997). Structure immunoreactive with porcine NPY in the caudal neurosecretory system of several fishes and cyclostomes. *Zool. Sci.* **14**, 665-669.

Pandey, A. C. (1981). Caudal neurosecretory system of a fresh water percid teleost, *Colisa fasciatus* (Bl. and Schn.): a histochemical study. *J. Hirnforsch.* **22**, 479-480.

Pandey, A. C. (1989). A histoenzymological investigation of the caudal neurosecretory system in six species of freshwater teleosts: a comparative study. *Gegenbaurs. Morphol. Jahrb.* **135**, 619-625.

Renaud, L. P. and Bourque, C. W. (1991). Neurophysiology and neuropharmacology of hypothalamic magnocellular neurons secreting vasopressin and oxytocin. *Prog. Neurobiol.* **36**, 131-169.

Stern, J. E. and Armstrong, W. E. (1995). Electrophysiological differences between oxytocin and vasopressin neurons recorded from female rats *in vitro*. *J. Physiol.* **488**, 701-708.

Vasilyev, D. M. and Barish, M. E. (2002). Postnatal development of the hyperpolarisation-activated excitatory current I_H in mouse hippocampal pyramidal neurons. *J. Neurosci.* **22**, 8992-9004.

Winter, M. J., Hubbard, P. C., McCrohan, R. J. and Balment, R. J. (1999). A homologous radioimmunoassay for the measurement of urotensin II in the flounder, *Platichthys flesus. Gen. Comp. Endocrinol.* **114**, 249-256.

Winter, M. J., Ashworth, A., Bond, H., Brierley, M. J., McCrohan, C. R. and Balment, R. J. (2000). The caudal neurosecretory system: control and function of a novel neuroendocrine system in fish. *Biochem. Cell. Biol.* **78**, 193-203.

Yamada, C., Owada, K., Ichikawa, T., Iwanaga, T. and Kobayashi, H. (1986). Immunohistochemical localisation of urotensins I and II in the caudal neurosecretory neurons of the carp *Cyprnus carpio* and the sharks *Heterodontus japonicus* and *Cephaloscyllium umbratile*. *Arch. Histol. Jpn* **49**, 39-44.

Yulis, C. R., Garcia, M. E. and Rodriguez, E. M. (1990). The caudal spinal cord of coho salmon (*Oncorhynchus kisutch*): immunocytochemical evidence of a 'caudal serotoninergic system'. *Cell Tiss. Res.* **259**, 543-550.

Zaninetti, M., Blanchet, C., Tribollet, E., Bertrand, D. and Raggenbass, M. (1999). Magnocellular neurons of the rat supraoptic nucleus are endowed with functional acetylcholine receptors. *Neurosci.* **95**, 319-323.

Zaninetti, M., Tribollet, E., Bertrand, D. and Raggenbass, M. (2002). Nicotinic cholinergic activation of magnocellular neurons of the hypothalamic paraventricular nucleus. *Neurosci.* **110**, 287-299.