SECTION 1A

Magnocellular Neuroendocrine Neurons: Properties and Control of Vasopressin and Oxytocin Neurons

CHAPTER 1

Electrophysiology of Magnocellular Neurons *In Vivo*

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1.1 Introduction

Neuroendocrinology is the study of things that matter: stress and appetite, metabolism, body rhythms, growth, and all aspects of reproduction from the reproductive cycle, through sexual behavior, pregnancy and parturition, to lactation and maternal behavior—things that matter for our health and happiness. However, neuroendocrine systems are also influential model systems for neuroscience generally, because of their unique value as "windows on the brain." The products of neuroendocrine systems can be measured relatively easily, and their effects are, with wit and persistence, determinable. In consequence, the neuronal activity that gives rise to those products is *interpretable* to a degree that can only be envied by colleagues in most other areas of neuroscience. If we ask of any neuron in the brain, what does it really *do*, the answers are often frustratingly incomplete: even if we know how it responds to stimuli, what it makes and where it projects, we may still not know what it does that matters to the behaving organism. By contrast, for the magnocellular vasopressin and oxytocin neurons of the hypothalamus, we can know much of what they do even before we know how they do it. All of these neurons project to the posterior pituitary gland, and what they secrete from there is measurable in the blood, and has measurable consequences for important physiological functions.

Neuroendocrinology began as the study of the secretion of peptides from neurons into the blood, and evolved to be also the study of secretion of peptides within the brain. In both aspects, electrophysiological recordings from single neurons *in vivo* have been fundamental in defining the physiological significance of mechanisms that have been established by cellular and molecular studies *in vitro* (Figure 1.1).

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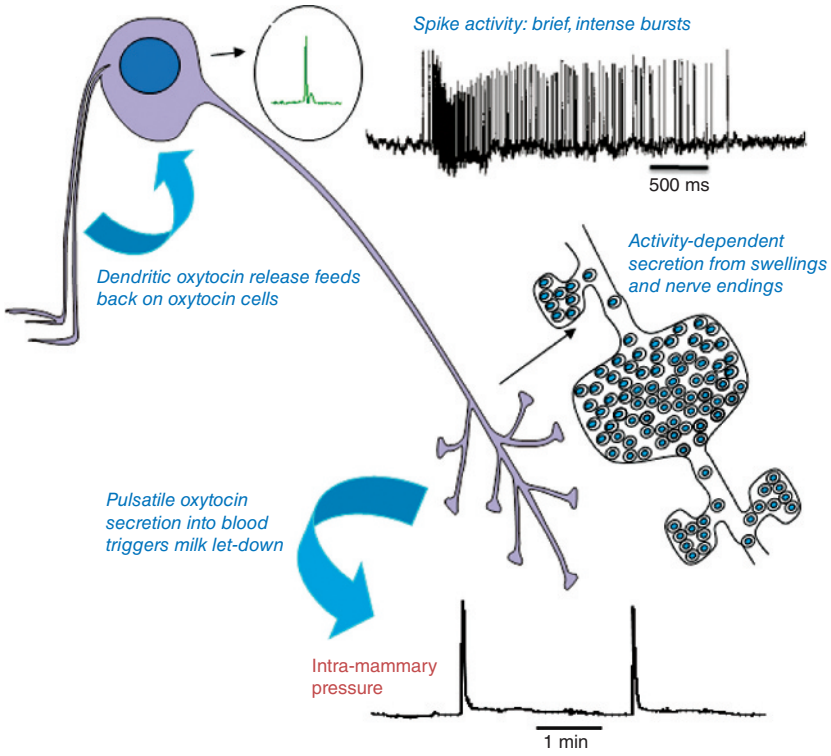

Figure 1.1 The milk-ejection reflex. The reflex was uncovered by electrophysiological studies *in vivo*. In response to suckling, oxytocin cells discharge intermittently in brief synchronized bursts that evoke secretion of pulses of oxytocin, which induce abrupt episodes of milk ejection. Dendritically released oxytocin facilitates the bursting.

1.2 Opening the window on the brain

Single-cell recording has strengths and weaknesses—and *in vivo* studies have particular problems as well as certain unique advantages. Because *in vivo* electrophysiology requires an extensive and prolonged investment in skills and expertise, it is wise to understand the nature of the investment that is involved, as well as the potential returns and the likely limitations.

The window on the brain afforded by the magnocellular system was opened by Wakerley and Lincoln (1973), when they used a technique to enable the magnocellular neurosecretory neurons to be rigorously interrogated electrophysiologically. There had been earlier attempts to study these neurons electrophysiologically, but despite careful stereotactical control, these recordings came from a mixture of neuroendocrine and non-neuroendocrine cells. This heterogeneity subverted interpretation: the breakthrough came from the ability to identify individual neurons as

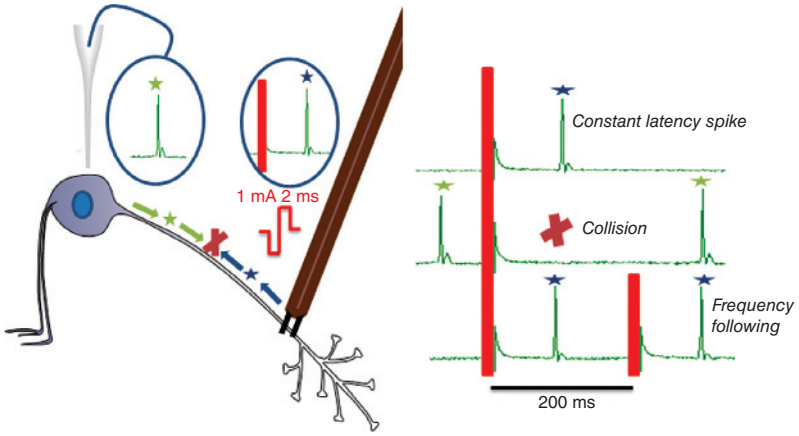

Figure 1.2 Antidromic identification. A stimulating electrode that is placed on the axon of any neuron may be used to trigger a spike that is propagated both orthodromically (green stars), toward the axonal endings, and *antidromically* (blue stars), toward the cell body. A recording electrode at the cell body will record the antidromic spike at a fixed latency following the stimulus—a latency that reflects the conduction velocity and the axonal length. In general, a stimulus pulse might evoke a spike that is generated by monosynaptic excitation, which would also arise at a nearly constant latency. Antidromic spikes can be distinguished from such orthodromic spikes by two additional tests: *frequency following* and the *collision* test. Antidromic spikes will be generated (i.e., will follow) each of a short train of stimulus pulses presented at a high frequency (50–100 Hz); these spikes will maintain a near-constant latency (there is a slight prolongation of latency with each successive pulse). A longer train of stimuli will result in fractionation of the antidromic spike—as the soma becomes refractory to antidromic stimulation, antidromic invasion is progressively delayed and may intermittently fail, while the smaller initial segment spike, which is normally hidden within the soma spike, will be preserved and become visible as a notch on the rising phase of the antidromic spike. However, antidromic spikes will not invade either the initial segment or the soma when they are extinguished by collision with a spontaneous, orthodromically propagated spike. This collision (red X) occurs when an antidromic stimulus pulse immediately follows a spontaneously generated spike—the descending spontaneous orthodromic spike meets the ascending antidromic spike along the axon, and both are extinguished by this collision.

neuroendocrine neurons immediately and unequivocally. That breakthrough came with the introduction of *antidromic identification* (Figure 1.2).

Because magnocellular neurons project to the pituitary, a stimulating electrode placed on the neural stalk can be used to trigger action potentials (spikes) in their axons, and these spikes can be detected at the soma, after antidromic (i.e., backward) propagation, as spikes that follow each stimulus at a constant latency. The rat supraoptic nucleus contains only two types of neuron—magnocellular oxytocin neurons and magnocellular vasopressin neurons, so every neuron recorded from this region that can be antidromically stimulated can be identified as one of these two types. Once Wakerley and Lincoln began to apply this approach, they saw that these

two cell types could be distinguished by other tests. These tests were refined by many others, leading to an extensive battery of tests that now allows us to talk of *identified oxytocin neurons* and *identified vasopressin neurons*.

From the ability to identify oxytocin and vasopressin neurons *in vivo* came the cornerstones of our understanding of the physiology of these systems. Wakerley and Lincoln (1973) defined the milk-ejection reflex, as it is known in all good text books (Figure 1.1). From their studies came the realization that, in response to suckling, oxytocin is not released continuously, but intermittently—in pulses that result from the brief intense synchronized bursts of action potentials generated by the oxytocin neurons (Figure 1.3). From Francois Moos and her colleagues (see Richard

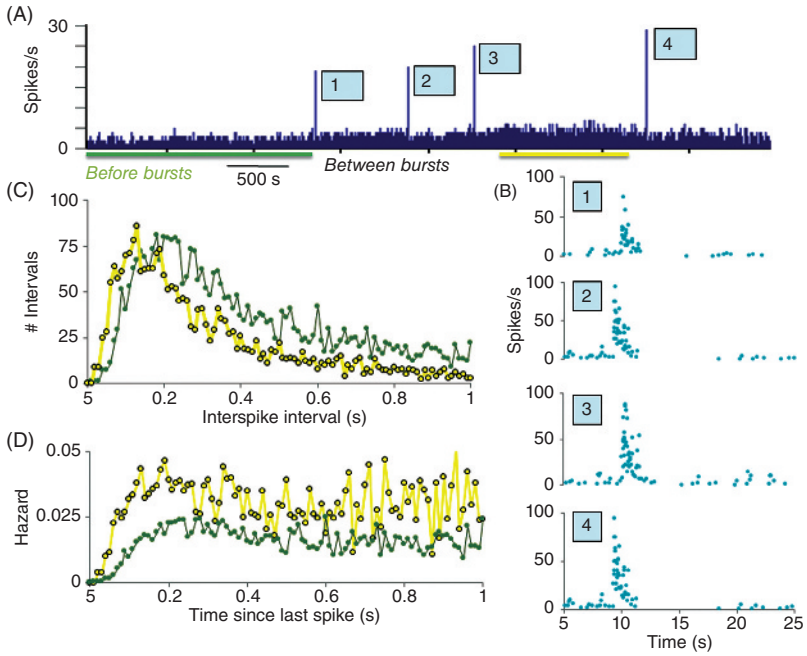

Figure 1.3 Milk-ejection bursts. Recording from an oxytocin cell in a urethane-anesthetized, lactating rat. Typically, oxytocin cells fire slowly and continuously, but, once the pups are applied, then brief intense bursts start to appear. (A) The first four bursts from one cell, progressively increasing in intensity. (B) Instantaneous frequency plots of these four bursts (the reciprocal of the interspike interval is plotted against spike time); note the consistency in the profiles of the bursts, which reveals their stereotyped structure, and the long quiet period following each burst. (C) Interspike intervals of the spontaneous activity before bursts (in green) and between bursts (in yellow). In this cell, the spontaneous activity is slightly elevated; typically, slow-firing cells become more active during suckling, while active cells become less active, but there is little change in the shape of the interspike interval distribution. (D) This is clearer in the hazard functions, which show a very similar shape except for the increase in hazard, which reflects an increase in mean firing rate.

et al., 1997), we learned that this reflex depends upon central release of oxytocin, and could be facilitated by injecting tiny amounts of oxytocin introcerebroventricularly (i.c.v.)—the first (and still the best) example of an indispensable physiological role of central peptide release. From Summerlee and Lincoln (1981), we learned that the milk-ejection reflex in conscious rats is the same as in anaesthetized rats—and that during parturition, oxytocin is again released in intermittent pulses as a result of similar synchronized bursting (Summerlee, 1981). From Jon Wakerley, Richard Dyball, and Dominique Poulain came the recognition that in response to osmotic challenge, vasopressin neurons fire not continuously, but phasically (Figure 1.4). This phasic firing is asynchronous among the vasopressin neurons, so that it leads to continuously maintained secretion—its significance is not in producing a phasic pattern of secretion, but in optimizing the efficiency of stimulus–secretion coupling at the nerve terminals. From Dyball and others, we learnt that oxytocin neurons are just as responsive as vasopressin neurons to osmotic pressure increases, leading to recognition of oxytocin's role (in many species) in regulating natriuresis. These pioneers laid the cornerstones of our current understanding, inspiring a wealth of hypotheses that have been pursued ever since by subsequent workers. Some of the questions that they posed were particular to these systems: What makes magnocellular neurons osmosensitive? Why is oxytocin released in response to osmotic stimulation? Many others were questions of general significance: Why do cells fire in bursts, and what are the mechanisms that underlie bursting? What mechanisms allow bursts to be synchronized? Why is it important that hormones are released in pulses? How can a peptide change the pattern in which cells fire? How is peptide release in the brain regulated? The mechanistic understanding that flowed from pursuit of the answers to these and related questions has changed our understanding of the brain in profound ways. Most neurons in the brain release one or more peptide messengers in addition to classical neurotransmitters, and these have a myriad of autocrine, paracrine, neuromodulatory and neurohormonal actions.

The ability to reliably identify neurons is critically important for electrophysiological studies *in vivo*: the brain is a large and diverse community, and adjacent neurons often have disparate functions, and this is especially true in the hypothalamus. In the paraventricular nucleus, for example, many different neuronal populations jostle together in distressing disharmony: there are magnocellular oxytocin neurons and vasopressin neurons like those in the supraoptic nucleus, but also populations of centrally projecting oxytocin and vasopressin neurons, neuroendocrine neurons that release thyrotropin-releasing hormone and corticotropin-releasing hormone, pre-autonomic neurons and interneurons. The supraoptic nucleus by contrast is wonderfully homogeneous, but at the margins there is still

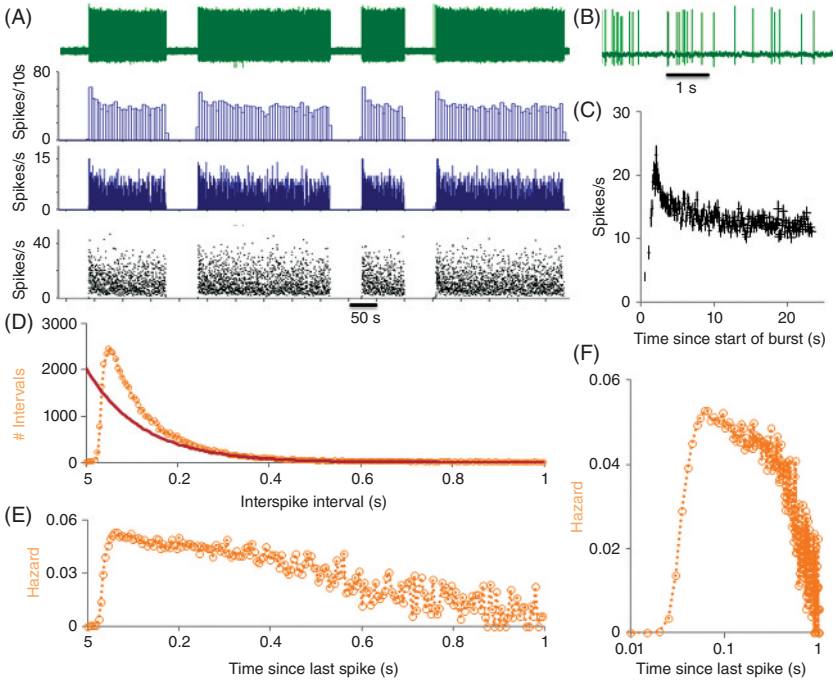

Figure 1.4 Phasic cells in the supraoptic nucleus. (A) Top, in green, the raw voltage trace of an extracellular recording of a phasically firing neuron. Below, in blue, the rate records in 10-s bins and in 1-s bins, and the instantaneous firing rate record, in black, which plots the reciprocals of the interspike intervals. Note that the apparent regularity of discharge in the 10-s bin ratemeter record is belied by the considerable variability of the instantaneous firing rate. (B) An expansion of the raw voltage trace displaying this irregularity. (C) Despite the irregularity of discharge on a short timescale, the phasic bursts have a very consistent structure, shown by the average shape of the start of bursts from the cell in B. Sixty-five successive bursts were analyzed, and the data show the mean arrival times of the first 200 spikes of each burst measured from the first spike in each burst, plotted against the mean instantaneous frequency (the crosses are the standard errors). (D) The interspike interval distribution for this cell; the red line is a single negative exponential fitted to data for intervals >300 ms (r^2 (0.95); note that shorter intervals lie above this line. (E) The corresponding hazard function: the hazard rises to a maximum at 60 ms and declines thereafter. This shape suggests that spike activity within a burst is strongly influenced by a sequence of spike-dependent hyperpolarizations and depolarizations, as expected from the superimposed influences of a large, transient HAP and a small, slower DAP. In addition, as shown in (F), where the hazard function is plotted on a log scale, there is a precipitous decline in hazard for intervals >500 ms. This reflects the fact that generally, phasic bursts contain few or no intervals exceeding 500 ms.

intermingling of magnocellular neurons and other non-neuroendocrine neurons. In hypothalamic slice preparations, the outline of the supraoptic nucleus is clearly visible: but reference to a brain atlas will make it clear that, in a 400-μm-thick coronal brain slice, it is difficult to find any orthogonal path for a recording electrode that will ensure that it encounters only magnocellular neurons. With the benefit of knowledge accumulated by many workers over many years, it is easy to find published examples of "supraoptic" neurons recorded *in vitro* that are, in hindsight, almost certainly not magnocellular neurons. Techniques for electrophysiological identification of magnocellular neurons *in vitro* are now more refined, and identification can be confirmed *post hoc* by filling cells and using immunohistochemistry. Thus far, intracellular recording of magnocellular neurons *in vivo* has proved so difficult that few have attempted it. There are approaches that enable extracellularly recorded neurons to be individually filled with dye (juxtacellular labeling)—but so far nothing has been published from these approaches for magnocellular neurons.

Accordingly, contemporaneous identification of magnocellular neurons *in vivo* requires precise placement of a stimulating electrode on the neural stalk. There are two ways of achieving this reliably. The first is that taken by Wakerley and Lincoln: they recorded from lactating rats, and in this preparation, a stimulating electrode sterotactically placed on the neural stalk from a dorsal approach will trigger secretion of a bolus of oxytocin that causes a sharp rise in intramammary pressure. Such confirmation of accurate placement is essential, but it restricts studies to studies of neurons during lactation. The alternative is to directly expose the neural stalk and supraoptic nucleus by transpharyngeal (ventral) surgery. Using this approach, we can record from a single identified cell for several hours, allowing repeated testing with drugs applied either intravenously (i.v.) or i.c.v. to either the third ventricle or (with a cannula implanted dorsally) to a lateral ventricle. It also is compatible with simultaneous microdialysis of the nucleus, which allows one to administer drugs directly to the nucleus or to collect samples for measurement of dendritic peptide release, and can be combined with electrical stimulation of afferent pathways. The ventral approach, however, is not compatible with the milk-ejection reflex.

1.3 The milk-ejection reflex

When pups suckle, magnocellular oxytocin cells in the maternal hypothalamus discharge a burst of spikes every 5–10 min (Figure 1.1), resulting in an abrupt milk let-down. Each burst lasts just 1–3 s, and every oxytocin cell will burst within about 500 ms of one another. Peak instantaneous firing rates are attained within a few spikes from the onset, and can

briefly reach 200 Hz (instantaneous frequency; bursts typically contain 50–100 spikes in 0.5–1.5 s), with interspike intervals of 5–10 ms. Between bursts, interspike intervals of <30 ms are almost never seen, marking out the burst discharge as wholly exceptional. After the burst, the cells typically fall silent for several seconds before they resume normal levels of spontaneous activity (Figure 1.3). The mechanisms underlying the generation and synchronization of bursts are now reasonably well understood, and involve dendro-dendritic communication between the magnocellular neurons.

Suckling releases oxytocin from the magnocellular neuron dendrites (Figure 1.1), this depolarizes the oxytocin cells and triggers Ca^{2+} release from intracellular stores, and these effects stimulate even more oxytocin release. After i.c.v. injection of as little as 1 ng of oxytocin, the bursts are more frequent and more intense, while i.c.v. injections of oxytocin antagonists, or microinjection into just one supraoptic nucleus, have the opposite effect—they block the reflex. Thus, the reflex depends upon endogenous oxytocin release from magnocellular neuron dendrites, making this the clearest known example of an essential role for a neuropeptide, and one of the few neurobiological examples of positive feedback.

1.3.1 Vasopressin cells and phasic firing

In response to dehydration or osmotic stimuli, and as a consequence of both of their intrinsic osmosensitivity and increased synaptic input, many vasopressin cells fire in a distinctive phasic pattern, with bursts mostly lasting 15–60 s and separated by silent periods mostly of 15–40 s, and with an intraburst firing rate of typically 4–10 Hz (Figure 1.4). Not all vasopressin cells fire phasically—in many conditions, most fire continuously, but it seems that many (and possibly all) can and do exhibit this mode of firing in some circumstances, such as dehydration. This pattern has attracted considerable attention; many neurons fire in bursts of spikes, but few have a bursting period as long as that of vasopressin cells. Much of what we know about the mechanisms underlying phasic firing has come from studies *in vitro*, but it is important to note that while phasic firing is observable *in vitro*, there are important differences between *in vitro* and *in vivo* phasic firing, differences that apparently arise from the fact that *in vitro* preparations are largely deafferented (Sabatier *et al.*, 2004). The loss of synaptic input has several consequences: many vasopressin cells in slice preparations (see Armstrong *et al.*, 2010) are silent unless the cells are exposed to extrinsic depolarization, and their input resistance is much higher because fewer ligand-gated ion channels are open. The elevated input resistance alters passive membrane properties, and exaggerates voltage changes that arise in response to imposed conductance changes; voltage-dependent conductances are likely larger and slower *in vitro* than *in vivo*. However, the

basic burst generating mechanisms are intact. If vasopressin cells *in vitro* are maintained at a resting potential close to spike threshold, then spikes will be followed by a depolarizing afterpotential (DAP) that triggers further spikes—thus spiking is regenerative *in vitro* (in slice preparations), and sustains a plateau potential, with bursts characterized by relatively regular spike discharge. As the burst progresses, the DAP is inactivated, terminating the burst. However, *in vivo*, spiking is not regenerative, but depends on afferent input. Fluctuations in excitatory input trigger spikes randomly, and the post-spike DAP enhances the probability of excitatory postsynaptic potentials (EPSPs) triggering spikes, and accordingly, spiking within bursts is very stochastic.

A curious feature of phasic cells is that they act as bistable oscillators—they have two stable states, an active state and a quiescent state, and small perturbations can flip a cell from either state into the other. Thus the same transient stimulus can either activate a phasic cell if it is silent or inhibit it if it is active (Figure 1.5). The significance of phasic firing thus lies not in the

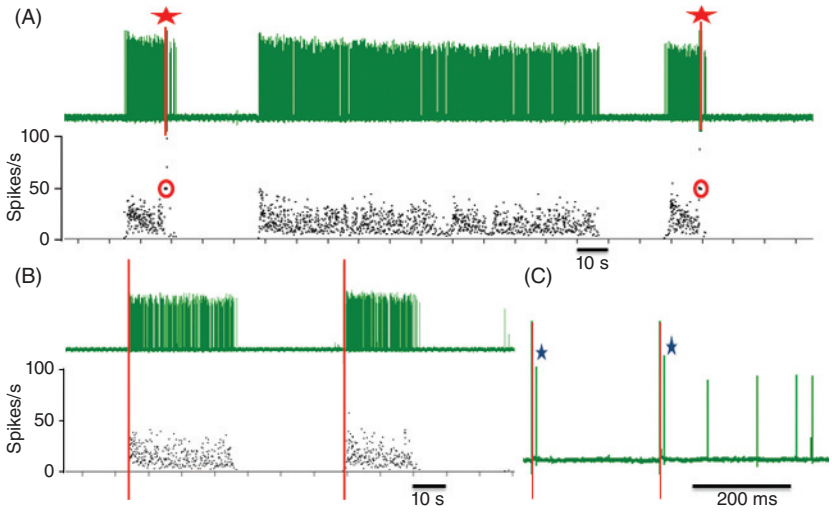

Figure 1.5 Vasopressin cells as bistable oscillators. Extracellular recording of a phasic neuron from the supraoptic nucleus of a urethane-anesthetized rat: voltage traces are shown in green above instantaneous frequency plots. Stimuli applied to the neural stalk evoke antidromic spikes that invade the cell bodies of the magnocellular supraoptic neurons. In (A), antidromic stimuli were applied during the bursts (red stars, lines and circles). Short trains of stimuli at 50 Hz were applied—note how the bursts are arrested, after a brief delay. In (B), stimuli were applied during the silent periods between bursts (red lines)—note how just two stimuli trigger bursts of activity. (C) Expansion of the record of the first episode of stimulation shown in (B); the blue stars mark the antidromic spikes evoked by each of two stimulus pulses, the artifacts from which are overlain by the red lines.

phasic patterning of information—this patterning is lost in the output from the pituitary gland because phasic cells discharge asynchronously. Instead it appears that phasic firing optimizes the efficiency of stimulus–secretion coupling at the nerve terminals.

1.4 Osmotic responses

Both oxytocin and vasopressin cells are excited by increased plasma osmotic pressure (and equally strongly, so this alone cannot distinguish vasopressin cells from oxytocin cells). An early study of Brimble and Dyball (1977) used intraperitoneal (i.p.) injection of hypertonic saline (1 mL of 1.5 M NaCl) as a standard stimulus, which raises plasma osmotic pressure by ~12 mOsm/kg over about 20 min; as the kidneys are nonfunctional under urethane anesthesia, this is a maintained increase. A problem is that i.p. injections can activate pain pathways, and there is often an initial transient response to i.p. injections that seems to be independent of plasma osmotic pressure. A better alternative is therefore slow i.v. infusion. When hypertonic saline is given in this way, there is an initial step rise in plasma sodium concentration depending on the concentration and rate of infusion, and thereafter, plasma sodium concentration rises linearly while the infusion continues. Thus in Leng *et al.* (2001), after infusion of 4.3 mL of 1 M NaCl over 60 min in urethane-anesthetized rats, plasma $[Na^+]$ increased from 146 to 165 mM, and plasma osmolality from 296 to 334 mOsm/L. Plasma $[K^+]$ also rose (from 3.3 to 4 mM), consistent with extensive cell shrinkage and passage of intracellular electrolytes into the extracellular fluid compartment. Hematocrit fell from 44.5% to 40%, consistent with an 11% increase in plasma volume.

1.5 Responses to other stimuli

While both oxytocin and vasopressin cells are activated by osmotic stimuli, two other stimuli have been used extensively to discriminate between them *in vivo*:

1 If 10 μg phenylephrine is injected i.v. (in 0.1 mL physiological saline), it produces a large (40–60 mm Hg) and abrupt transient increase in blood pressure. This will interrupt the firing of a phasic cell if applied in mid-burst, but has little effect upon most continuously firing cells (Harris *et al.*, 1975). In some continuously active cells, however, baroreceptor stimulation will interrupt the activity for ~20 s, and activity resumes with the abrupt onset typical of phasic bursts. This has led some authors to use baroreceptor stimulation for discriminating oxytocin cells from

vasopressin cells, but this stimulus has not been systematically studied in cells identified by the milk-ejection reflex. Furthermore, in the rat, vasopressin cells are relatively insensitive to baroreceptor stimulation. A large rise in blood pressure (over 50 mm Hg) must be induced, and even for phasic cells, the response may be unreliable: if baroreceptor stimulation is applied at the beginning of a burst it may fail to interrupt the burst. Nonetheless, baroreceptor stimulation may be useful in recognizing some vasopressin cells that are active but which do not exhibit phasic firing. This baroreceptor pathway is thought to involve GABA as a final inhibitory transmitter from neurons in the perinuclear zone dorsolateral to the supraoptic nucleus, and to involve a projection from the caudal brainstem to the diagonal band of Broca.

2 If cholecystokinin (CCK) is injected i.v. in rats (25 μg/kg in 0.1 mL physiological saline), it will produce a modest increase in the firing rate of oxytocin cells that lasts for 10–15 min (Renaud *et al.*, 1987; Figure 1.6). This increase is accompanied by an increased secretion of oxytocin. Injections of CCK inhibit most vasopressin cells for a similar duration (accompanied by reduced vasopressin secretion). Systemically injected CCK acts at CCK-1 receptors on afferent vagal neurons that innervate the stomach and duodenum, these in turn activate noradrenergic neurons of the A2 cell group in the nucleus tractus solitarii that project directly to magnocellular oxytocin neurons. How the inhibition of vasopressin neurons is mediated is not known.

Both of these stimuli can be given repeatedly with consistent effects. Experience with these stimuli and functional identification with the milk-ejection reflex has led to refined ways of distinguishing between the cell types based on statistical features of their discharge patterning.

Many other stimuli are known to activate magnocellular neurons under urethane anesthesia—but are not so helpful in discriminating between oxytocin cells and vasopressin cells. Secretin for example activates both cell types when injected at very low doses (as little as 1 μg injected i.v.; Figure 1.6). The physiological significance of this is as yet unknown. The response to CCK seems likely to be associated either with the appetite-suppressing effects of centrally released oxytocin, or with the natriuretic effects of peripherally secreted oxytocin: CCK is secreted from the duodenum in response to food intake and triggers satiety.

The ability to identify oxytocin and vasopressin neurons *in vivo* has allowed systematic analysis of the role of afferent pathways in controlling their activity. In the case of the activation of oxytocin neurons by CCK, for example, we now know that this stimulus begins as activation of CCK-1 receptors on afferent vagal nerve endings that innervate the gastrointestinal tract. This pathway relays in the nucleus tractus solitarii of the caudal brainstem, from where noradrenergic neurons of the A2 cell group

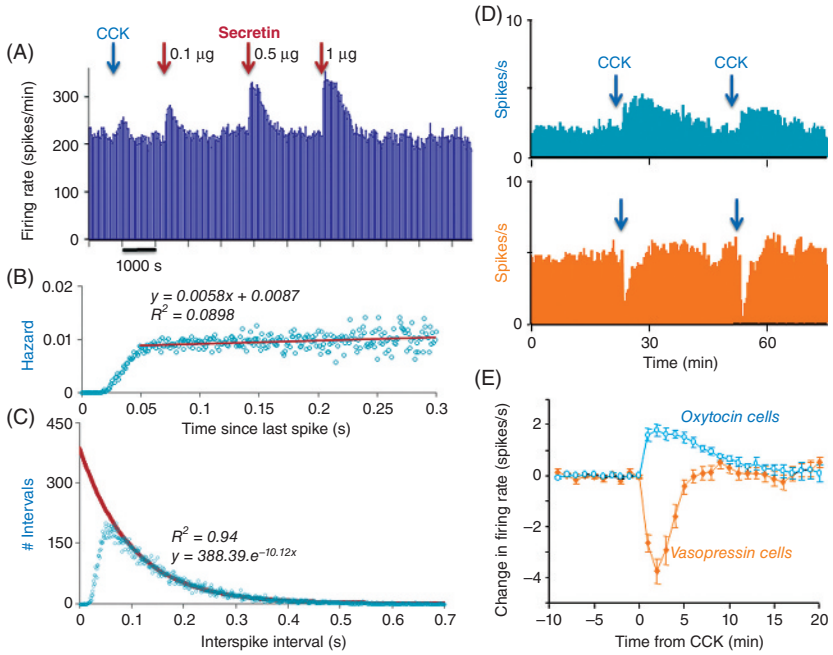

Figure 1.6 Responses of supraoptic neurons to gut-related peptides given i.v. (A) Responses of an oxytocin cell in a urethane-anesthetized rat to oxytocin and secretin given i.v. (from Velmurugan *et al.*, 2010). The excitatory response to cholecystokinin (CCK) is typically small (0.5–1 spikes/s)—larger responses are evoked by secretin, but secretin injections also activate vasopressin cells. (B) The hazard function from the cell shown in A: the function has the shape that is characteristic of oxytocin cells, rising slowly to a relatively constant plateau level after about 50 ms. The plateau level is shown in red as the line of best fit to hazard data from 50 ms onward. (C) The corresponding interspike interval distribution; in this case, the red line represents a single negative exponential fit to intervals >50 ms. (D) Data from a simultaneously recorded oxytocin neuron (in blue) and a continuously active vasopressin neuron (in orange). Two sequential injections of CCK elicited repeatable excitation of the oxytocin cell and inhibition of the vasopressin cell. (E) Averaged responses to CCK of oxytocin cells and continuously active vasopressin cells. Modified from Sabatier *et al.* (2004).

that co-express prolactin-releasing peptide, (and other peptides including enkephalin) project directly to magnocellular oxytocin neurons. This projection is modulated by opioids: μ-opioid receptors are present presynaptically, and retrodialysis of μ-agonists onto the supraoptic nucleus blocks CCK-evoked noradrenaline release at that site (Onaka *et al.*, 1995).

The projections from the caudal brainstem thus mediate gastric-related stimuli as well as cardiovascular stimuli and stimuli arising from the reproductive tract. Projections from anterior regions (the subfornical organ, organum vasculosum of the lamina terminalis (OVLT) and the nucleus

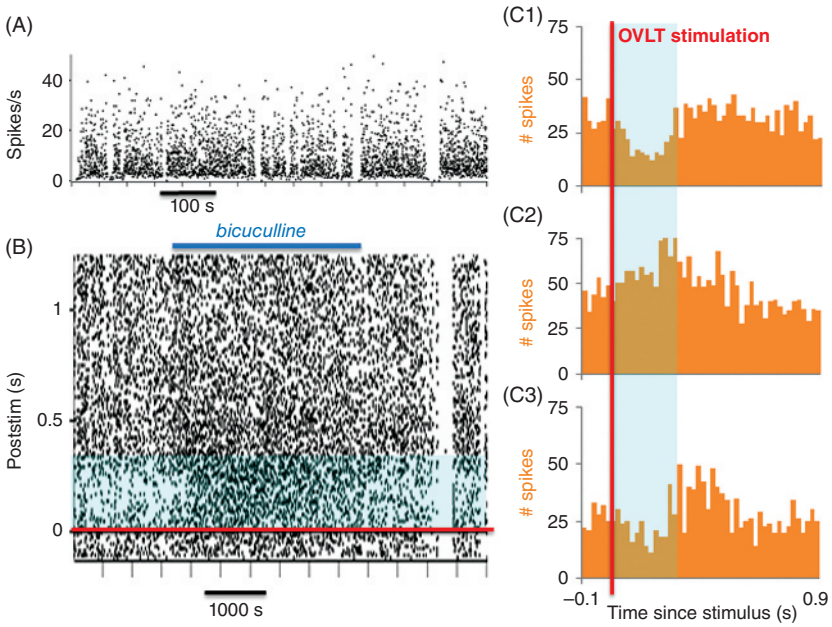

Figure 1.7 Effects of stimulation of the organum vasculosum of the lamina terminalis (OVLT) on a phasic supraoptic neuron *in vivo*. (A) Extract of recording from a phasic neuron in the supraoptic nucleus of a urethane-anesthetized rat; instantaneous frequency plot. (B) Raster plot showing effects of stimuli applied to the OVLT (red bar). The effects of the stimuli are visible in the blue shaded region of the raster plot, and quantified in the post-stimulus time histogram in **C1**. Initially, OVLT stimulation is inhibitory, but after application of the $GABA_A$ receptor antagonist bicuculline to the supraoptic nucleus by retrodialysis, there is (a) an increase in spontaneous activity; (b) a loss of the inhibitory effects of stimulation; and (c) an unmasking of excitatory effects of stimulation (**C2**). After washout of bicuculline, the inhibitory response to OVLT stimulation returns (**C3**) (see Leng *et al.* (2001), for details).

medianus) convey osmotic information and signals arising from several blood-borne hormones, including angiotensin: these inputs involve both excitatory components and inhibitory components (Figure 1.7). There is an interesting input from the suprachiasmatic nucleus relaying photoperiodic information; our understanding of the significance of this is still unfolding. Finally, there are inputs from the arcuate nucleus (Figure 1.8), including from neurons that release the peptide α-melanocyte-stimulating hormone (α-MSH): this is an extremely interesting pathway as α-MSH stimulates dendritic oxytocin release and induces expression of c-*fos* in magnocellular oxytocin neurons, apparently as a consequence of its ability to mobilize intracellular calcium stores in oxytocin cells. However, at the same time, it inhibits the electrical activity of oxytocin cells (Figure 1.9), and this is apparently the consequence of evoked release of endocannabinoids, which

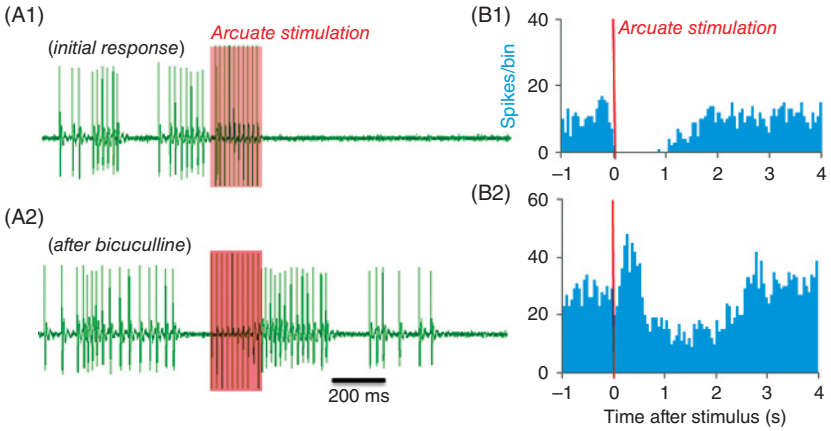

Figure 1.8 Effects of arcuate nucleus stimulation on supraoptic neurons. Electrical stimuli applied to the arcuate nucleus inhibit most supraoptic neurons with a short latency; **A1** shows an extract of the original recording, **B1** shows the post-stimulus time histogram (5 ms bins) constructed from 300 repetitions. This inhibition can be blocked completely if the $GABA_A$ receptor antagonist bicuculline is applied to the supraoptic nucleus by microdialysis (**A2** and **B2**). Blocking this inhibition unmasks an excitatory effect of stimulation, the mediator of which is at present unknown (see Ludwig and Leng (2000) for full details).

act to presynaptically suppress afferent glutamatergic pathways, including from the OVLT. Thus, magnocellular oxytocin neurons can release oxytocin either centrally from their dendrites or peripherally from their nerve endings in the pituitary, depending on exactly how they are activated—reminding us also that electrophysiology alone cannot define the behavior of the cells.

1.6 The future

Although we now have answers to many of the questions posed by the pioneers of *in vivo* electrophysiology, we should not think that we now know enough about the physiology to concentrate only on mechanism. On the contrary, there are still massive gaps in our physiological understanding, gaps that may still hide fundamentally important physiological principles. For one example, the textbooks tell us of vasopressin's role in water retention, and its regulation by osmotic pressure and blood volume. It now seems that perhaps a third major controller has gone unnoticed until recently—the vasopressin cells are also exquisitely sensitive to temperature—is this a reflex to conserve water in conditions of evaporative water loss? For another, the oxytocin system is exquisitely sensitive to activation by a wide range of hormones released from the

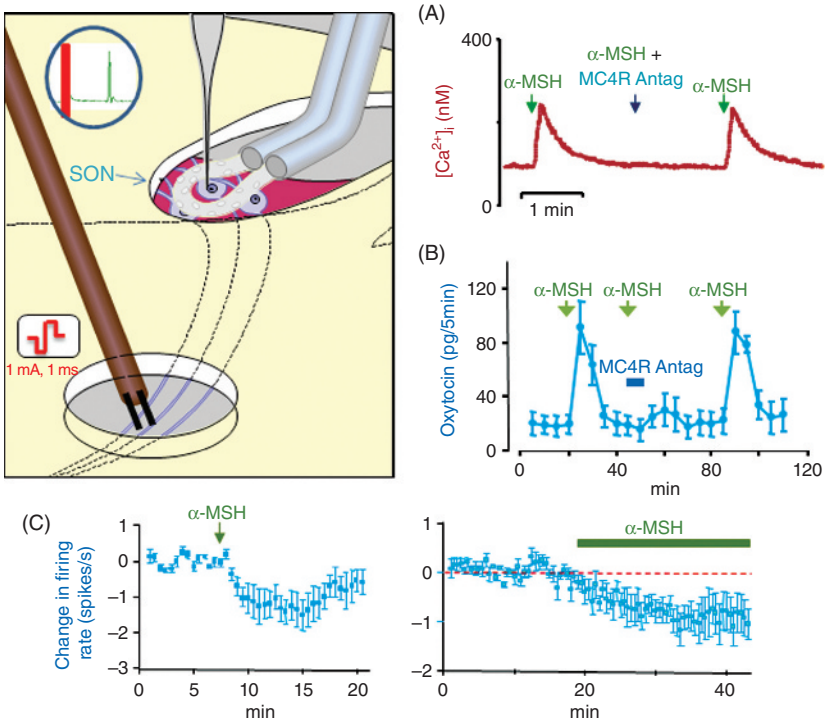

Figure 1.9 Dissociation between electrical activity and dendritic peptide release. Oxytocin neurons express melanocortin 4 (MC4) receptors, which mediate their responses to α-melanocyte-stimulating hormone (α-MSH), a peptide released from the pro-opiomelanocortin neurons of the arcuate nucleus that project directly to the supraoptic (SON) and paraventricular nuclei. (A) Exposure to α-MSH (A) triggers an increase in intracellular calcium concentration that results from a mobilization of intracellular stores; and (B) evokes dendritic oxytocin release both effects of which are blocked by the MC4 receptor antagonist (MC4R). However, α-MSH given intracerebroventricularly (C, left) or by retrodialysis directly to the supraoptic nucleus (C, right) inhibits the activity of identified oxytocin neurons (see Sabatier *et al.* (2003) for details).

gastrointestinal system—does the magnocellular oxytocin system have a role in energy intake and energy balance or in regulating the gastrointestinal tract? These two are latent physiological stories, still to be fully elaborated, both triggered by observations made *in vivo*.

Electrophysiological studies *in vitro* are not an alternative to studies *in vivo*; these are complementary undertakings, with fundamentally different purposes. *In vitro* preparations come with the ability to precisely control the intracellular and extracellular environment of neurons, which is essential for unravelling detailed membrane mechanisms and intracellular processes, but they come without the networks and physiological control systems that give these mechanisms meaning. *In vivo* preparations come

with a host of problems and difficulties, but are and will remain the ultimate reference for physiological significance.

1.7 Technical appendix

1.7.1 The milk ejection preparation: Technical details

The reflex is not observed with all anesthetics, or if anesthesia is too light or too deep. We anesthetize rats with i.p. injection of 1.1 g/kg urethane (ethyl carbamate, given as a 25% w/v solution in water); a lower dose (0.8 g/kg) with xylazine (10 mg/kg given intramuscularly as a 2% solution) can produce more frequent milk ejections—more frequent than seen in conscious rats. The reflex can also be facilitated by the β-adrenoreceptor antagonist propranolol (250 μg/kg i.p. or i.v.) given after surgery (Tribollet *et al.*, 1978), and further injections may be given if the reflex is slow to be established. We use rats at day 8–10 of lactation; after day 12, the eyes of the pups are open and they are less likely to suckle continuously. The mother is separated overnight from her litter to ensure that the mammary glands are full of milk, and that the pups are hungry. We cannulate an inguinal mammary gland by gripping the end of the teat with a forceps and cutting the skin to expose the duct. The duct is separated from supporting tissue, and is nicked with fine scissors. Gentle pressure at the base of the gland will fill the duct with milk and cause a little to be expressed through the nick. A polythene catheter (1 mm outer diameter filled with 0.15 M Na acetate) is introduced into the duct at the nick, tied firmly in place, and connected to a pressure transducer. A bolus injection of 0.5 mU oxytocin i.v. will cause an abrupt but transient increase in intra-mammary pressure of ~10 mm Hg, and a good preparation will show intramammary pressure increases to as little as 0.1 mU oxytocin (Video 1.1).

The rat is then placed in a stereotaxic frame with the skull level between the bregma and lambda. The supraoptic nucleus lies 1.5–2 mm lateral to the midline and about 0.5 mm posterior to the bregma, so a hole is drilled in the skull to allow a recording electrode to be introduced at these coordinates; the nucleus is encountered at about 8.5 mm from the dorsal surface of the brain. A second hole is drilled to place a fine injection cannula filled with l mU/μL oxytocin (1 mU = 2 ng) in a lateral ventricle, and a third is drilled in the midline to place a concentric bipolar stainless steel stimulating electrode on the neural stalk. This is drilled about 1.5 mm caudal to the lambda, as this region is free of the midline sinus. The stimulating electrode is introduced at an angle calculated to intersect with the stalk at a depth of about 9 mm. After a depth of 8.5 mm, the electrode is advanced in steps of 0.1 mm, between which a train of 90 pulses at 50 Hz is applied (matched biphasic square-waves, 1 ms duration, 0.5 mA peak-to-peak). At the

Video 1.1 Suckling rat pups responding to the release of oxytocin, producing milk ejection into the mammary duct of a lactating mother. Note the stretch reflex (outstretched limbs) indicative of vigorous suckling and retrieval of milk, especially visible in the pup in foreground.

optimum position, this train elicits a rise in intramammary pressure similar to that following i.v. injection of 0.5 mU oxytocin, at a delay of ~12 s. We apply the pups to the nipples immediately after completing the surgery; it can take some time for the pups to "work" the nipples and attach strongly to them, and it is unusual to see any milk ejections at this time. We then remove the pups again after all of the nipples have been "worked" by the pups and after all have become enthusiastic to suckle, and reapply them at ~2 h after the end of all surgery: now the pups should reattach very quickly, and in a good preparation, the reflex should start within 20–30 min. The reflex depends upon how many pups are suckling: we try to keep at least nine sucking continuously. Once the reflex is established, milk ejections typically occur every 5–10 min for 2–3 h. After the first few, relatively small, milk ejections, a typical milk ejection gives an intramammary pressure response equivalent to that evoked by i.v. injection of 0.5 to l mU oxytocin.

Injections of 1 μL oxytocin i.c.v. (into a lateral ventricle) will facilitate the reflex markedly in most rats, and can be repeated at intervals of ~30 min (Freund-Mercier and Richard, 1984); i.v. injections of oxytocin have no such effect, as oxytocin does not cross the blood–brain barrier in appreciable amounts. The reflex is catastrophically impaired if there is much blood loss: if the mean arterial blood pressure falls below 100 mm Hg, or if respiration is labored, then milk ejections will not occur. The more traumatic the surgery, the less likely it is that animals will milk eject.

The surgery requires an operating microscope with a long working distance, using the highest magnification. It also requires good eyes and steady hands and practice. The key to success is speed and precision.

1.7.2 Ventral surgery: Technical details

In a lactating rat, we can place a stimulating electrode precisely on the neural stalk because of the ability to verify correct placement by monitoring the intramammary response to stimulation. In non-lactating rats, similar certainty requires direct exposure of the neural stalk by transpharyngeal (ventral) surgery.

The rat is anesthetized deeply by i.p. injection of 1.25 g/kg urethane. Urethane gives stable, prolonged anesthesia, and has been the anesthetic of choice since it was first recognized that the milk-ejection reflex is essentially identical in urethane-anesthetized rats as in conscious rats. It has one recognized disadvantage: under urethane, both oxytocin neurons and vasopressin neurons are relatively hyperactive as a result of elevated plasma osmolality, and for the same reason, the expression of c-*fos* is markedly elevated in magnocellular neurons, making it impractical to combine electrophysiology with studies of Fos expression in the same preparation.

We begin by cannulating the trachea, and placing other cannulae as needed in the femoral vein and/or a branch of the jugular vein for administering drugs, and a femoral artery if needed to record blood pressure or withdraw blood samples. The vascular cannulae should be filled with isotonic saline—heparin should not be used until surgery is complete. The rat is then placed supine in a head holder fixed to the surface of a vibration isolation table, with the upper incisors raised 5 mm above the interaural line. The ranii of the mandible are separated with scissors, avoiding cutting either the skin of the lower jaw or the tongue, and the tongue is pulled between the lower incisors, and held in place with a retractor. Two more retractors are used to retract the lower incisors laterally, "gaping" the mouth, and revealing the soft palate to view. Using an operating microscope, the soft palate is cauterized with a fine cautery knife along its visible extent in the midline, and about 3 mm of the anterior portion of the hard palate is removed with a fine dental drill (after stripping the soft tissue from it). The surgery thus far should involve little blood loss: the only major hazard is at the junction between the hard and soft palates where a large vessel crosses the midline, requiring careful use of a fine electrocautery knife; if the knife is too hot, the vessel will rupture. The presphenoid bone will now be visible, and two features are important to identify: the suture between the presphenoid and basisphenoid bones, which is just medial to the supraoptic nucleus, and a venous sinus within the basisphenoid bone, at the rostral end of the pharynx, which overlies the neural stalk. The sinus appears as a purple "smudge" across the sphenoid bone, and can be the

source of severe blood loss unless tackled appropriately. We drill a shallow hole in the midline, 2 mm caudal to the sinus, pack it with bone wax (Ethicon Inc.), and progressively extend it forward, packing it with bone wax at regular intervals. As the drilling breaks through into the sinus, the drill spins bone wax into it, blocking it. The hole is then drilled deeper, and the last thin layer of bone removed with watchmaker's forceps and a tiny "hook" formed from a bent needle to expose the rostral adenohypophysis and the neural stalk. Keep the exposure to the midline: extending it lateral engages blood vessels. The neural stalk should be visible through the dura: blood vessels converge at this site, giving an appearance that contrasts strongly with the pink tissue of the pituitary and the gray tissue of the median eminence rostral to the stalk.

To expose the supraoptic region, the lateral wing of the palatine bone and medial pterygoid plate are cleared of muscle, and drilled away to expose the trigeminal nerve bundle. The nerve is removed from above the supraoptic nucleus with two pairs of fine forceps, one to lift a portion of the bundle, the other to cut it and peel it away. Removing this nerve leaves the supraoptic region still obscured by a large venous sinus, which runs along the lateral margin of the sphenoid bone. This is compressed with small balls of tissue, which are wedged against the sphenoid bone rostrally and caudally; this packing must not involve compressing the brain—the pledgets must compact the sinus into the sphenoid bone. The very dense vasculature of the supraoptic region can be seen through the dura, contrasting with the white of the optic chiasm. Using a fine needle, a tiny hole is made in the dura over the supraoptic nucleus, and through this, a recording electrode is introduced. There are numerous hazards along the way: among these are an artery just lateral to the trigeminal nerve that must not be severed, so avoid the temptation to explore more laterally than is needed. There is a small artery that crosses the optic chiasm and penetrates the nucleus: avoid this.

Refinements of this approach include additionally exposing the arcuate nucleus or the region of the OVLT, two regions that are sources of afferent input to the supraoptic nucleus. We also commonly place a U-shaped microdialysis probe on the ventral surface of the supraoptic nucleus, in this case after extensively cutting into the dura. Normally, an extensive break in the dura renders the preparation very unstable, but placing a dialysis probe on the surface stabilizes it again very effectively. It is also possible to insert an injection cannula in the third ventricle if the neural stalk exposure is extended rostrally to expose the median eminence.

With practice, the basic surgery can be completed in 20–30 min, with a loss of only about 0.5 mL of blood, though much larger amounts can be lost from either the basisphenoid sinus or the junction between hard and soft palates if they are not tackled effectively. However, blood pressure after

surgery is normally only 60–80 mm Hg unless corticosteroids are given (which we do not do). The supraoptic neurons are very active—in normal virgin rats or male rats, oxytocin neurons fire at a mean rate of 3–4 spikes/s (with a large variation between cells), and vasopressin cells on average slightly faster, again with a large variation between cells; basal plasma levels of both oxytocin and vasopressin are typically 50–100 pg/mL—much higher than in conscious rats. The main reason for the elevated firing rate and secretion seems to be the elevated osmotic pressure that is a consequence of the urethane injection, which causes a plasma osmotic pressure typically about 20 mOsm/kg above normal levels.

1.7.3 Recording electrodes

We routinely use glass micropipettes that naturally break upon gentle wiping to give a tip diameter of 1–3 μm. These pipettes have an internal glass fiber for easy filling, and are filled with 0.9% NaCl. Do not be tempted to use hypertonic saline to fill them: there is no noticeable advantage from the reduction in electrode resistance, but recordings are much less stable as the neurons are directly excited by leakage of hypertonic solutions. Tungsten microelectrodes with tip diameters of 1–5 μm are a good alternative. We currently use a patch-clamp amplifier and record in current-clamp mode with minimal signal filtering, and we use a HumBug noise eliminator (Quest Scientific) to exclude any residual mains interference without altering the original signal. Spikes from single neurons can vary in shape and polarity depending on the mode of recording and the precise site of recording. Generally, though, spontaneous spikes from magnocellular neurons are relatively broad and predominantly monopolar, with no visible inflection on the initial rising phase that, in some neuron types, reflects the initial segment spike; they very often, however, have a distinct notch on the descending phase that apparently reflects spike propagation in the proximal dendrite (Mason and Leng 1984). If the electrode tip is broken to a diameter of ~5 μm, smaller spikes may be seen with reversed polarity; these apparently are mainly from afferent nerve fibers.

It is not true that large-diameter electrodes record exclusively from cell bodies. Recordings from single axons can be made readily from the neural lobe using electrodes broken to ~5 μm in diameter (Figure 1.10), but not with finer electrodes, and the amplitude of spikes recorded from the neurohypophysis is similar to those recorded in the supraoptic nucleus (Dyball *et al.*, 1988). Single axons may be identified orthodromically via a stimulating electrode placed upon the neural stalk: stimulus-evoked spikes occur at a constant-latency, but fail the collision test, and frequency following is intermittent, as propagation is unreliable in the terminal regions.

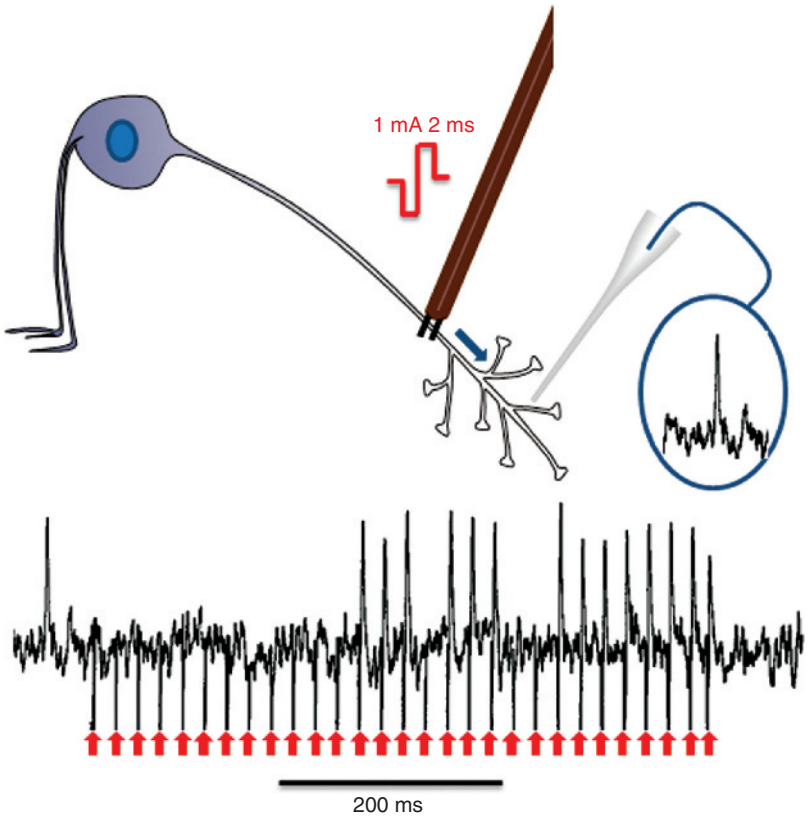

Figure 1.10 Recordings from the neurohypophysis. Extracellular recording of a single axon from the neural lobe in a urethane-anesthetized rat. Stimuli applied to the neural stalk evoke action potentials that are conducted orthodromically toward the nerve endings, and these can be detected as constant latency spikes (upward going deflections). Note, however, that spike propagation down the axons is not consistent—spikes commonly fail to invade the entire axonal arborization. However, high frequency stimulation (red arrows) results in more effective invasion, and this is thought to be part of the reason why milk-ejection bursts are so potent at stimulating oxytocin secretion. See Dyball *et al.* (1988) for full details.

1.7.4 Analysis of firing patterns

From the early experiments of Wakerley and co-workers, it was recognized that many supraoptic neurons fire in a phasic discharge pattern (Figure 1.4), especially after osmotic stimulation, and that these phasic neurons rarely respond to suckling and hence are likely to be vasopressin neurons. Objective criteria for quantifying phasic firing by automated analysis were soon developed. These analyses quantify burst duration, interburst interval ("silent" period) duration, intraburst firing rate, and activity quotient (the proportion of time active). Bursts can be identified automatically according

to the following criteria: a burst comprises at least 20 spikes, lasts for at least 3 s, contains no interspike interval of >1 s, and begins with an interspike interval of <800 ms. For phasic supraoptic neurons, such analyses typically partition >95% of recorded spikes into "bursts." Sabatier *et al.* (2004) described analysis of 83 phasic cells with a mean firing rate of 4.2 ± 0.2 spikes/s (range 0.9–10.7 spikes/s), a mean burst duration of 62 ± 6 s (range 10–360 s; median 42 s), and interburst (silence) duration of 37 ± 4 s (range 4–216 s; median 28 s). The mean intraburst firing rate was 7.1 ± 0.3 spikes/s (range 2.3–13.3 spikes/s; median 7.1 spikes/s). Interspike interval distributions were markedly skewed, with modes of 45 ± 1.5 ms (range 15–80 ms), and very few intervals of less than 20 ms (1.6% of 211,817 intervals).

Phasic cells are easy to recognize, but many vasopressin cells fire continuously, in a way that is at first sight much like oxytocin cells, though oxytocin cells (in virgin or male rats) fire more regularly that continuously active vasopressin cells. One way of measuring regularity is by the *coefficient of variation* of the interspike intervals, defined as the (standard deviation/mean interval) × 100%. For wholly random events (generated by a Poisson process), the coefficient of variation will be 100%. In the samples reported by Sabatier *et al.* (2004), the mean coefficient of variation of intraburst activity in phasic cells (excluding intervals >1 s) was 100.6 ± 1.9% ($n = 77$), similar to that of continuously active vasopressin cells *in vivo* (100.4% ± 5.4%, $n = 22$), and both were significantly higher than the coefficient of variation for oxytocin cells (75.1 ± 1.8%, $n = 23$). Other measures of interspike interval regularity (based on entropy) are very closely correlated with the coefficient of variation for both oxytocin cells and vasopressin cells, and, therefore, do not add anything independent of this. As well as being more regular, continuously firing vasopressin cells also tend to be more active than oxytocin cells, but there is considerable overlap in firing rates between cells, so this is not particularly helpful as a discriminant.

It is also possible to reliably discriminate between oxytocin cells and vasopressin cells *in vivo* by constructing hazard functions from their spontaneous discharge patterns. The spontaneous firing pattern of any neuron reflects: (a) the intrinsic membrane properties of that neuron; (b) afferent inputs perturbing those properties; and (c) recurrent (network) effects. Different populations of neurons vary considerably in their intrinsic properties, and analyzing spontaneous firing patterns is an important aid to cell identification. Oxytocin neurons display a conspicuous hyperpolarizing after-potential (HAP) that renders them relatively inexcitable for at least 25 ms after a spike—so it is only within milk-ejection bursts that intervals shorter than this are ever seen. The interspike interval distribution recorded from a period of spontaneous activity is unimodal, with a mode at 25–75 ms, at most, and the distribution of intervals greater than the mode that

can be well fitted by a single negative exponential. This is consistent with the interpretation that spikes in oxytocin cells arise randomly, except that they are constrained by the refractoriness imposed by the HAP. Trains of spikes in oxytocin cells are also influenced by a slower activity-dependent hyperpolarization—the afterhyperpolarization (AHP); its effects cannot be discerned from the interspike interval distribution, but can be discerned by more complex analysis of order effects.

The hazard function characterizes how the excitability of a cell changes following a spike, and is calculated (in 5-ms bins) by the formula:

$$(\text{hazard in bin}[t, t+5]) = (\text{number of intervals in bin } [t, t+5])/(\text{number of intervals of length} > t), \text{ for } t = 5, 10, 15 \ldots$$

If spike times were wholly random, then the hazard function would be a line parallel to the x (i.e., time) axis at a level that reflects the mean rate. For oxytocin cells, the hazard increases immediately after a spike from zero to a level at about 50 ms that thereafter remains approximately constant. However, for vasopressin cells, the hazard rises to a peak at about 50 ms, from which it subsequently declines; this reflects the fact that, in vasopressin cells, the HAP is generally followed by (at least two, fast and slow) DAPs.

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