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The Adaptive Brain: Glenn Hatton and the Supraoptic Nucleus

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Abstract

In December 2009, Glenn Hatton died, and neuroendocrinology lost a pioneer who had done much to forge our present understanding of the hypothalamus and whose productivity had not faded with the passing years. Glenn, an expert in both functional morphology and electrophysiology, was driven by a will to understand the significance of his observations in the context of the living, behaving organism. He also had the wit to generate bold and challenging hypotheses, the wherewithal to expose them to critical and elegant experimental testing, and a way with words that gave his papers and lectures clarity and eloquence. The hypothalamo-neurohypophysial system offered a host of opportunities for understanding how physiological functions are fulfilled by the electrical activity of neurones, how neuronal behaviour changes with changing physiological states, and how morphological changes contribute to the physiological response. In the vision that Glenn developed over 35 years, the neuroendocrine brain is as dynamic in structure as it is adaptable in function. Its adaptability is reflected not only by mere synaptic plasticity, but also by changes in neuronal morphology and in the morphology of the glial cells. Astrocytes, in Glenn's view, were intimate partners of the neurones, partners with an essential role in adaptation to changing physiological demands.

Keywords

oxytocin; vasopressin; hypothalamus; astrocytes; pituicytes; pituitary

Academic history

Neuroscience, and particularly neuroendocrinology, lost a great friend and pioneering investigator with the death of Glenn I. Hatton on 16 January 2009 (Fig. 1). At the time of his passing, Glenn was a Distinguished Professor in the Department of Cell Biology and Neuroscience at University of California, Riverside, where he had served as Chair, and as founding Director of the Neuroscience Program from 1992 to 2002. He had recently celebrated his 74th birthday.

Glenn hailed from Chicago, Illinois, and received his BSc from North Central College, Naperville, Illinois, in 1960 in Psychology. He then studied under Professor Larry O'Kelly at the University of Illinois, Urbana, where he received MA (1962) and PhD (1964) degrees in Psychology. After a very short postdoctoral position, Glenn became Assistant Professor of Psychology at Michigan State University in East Lansing in 1965, where he would remain for 26 years and become Distinguished Professor in 1986. He also served as Director of MSU's fledgling Neuroscience Program from 1978 to 1991.

Glenn's career began with behavioural and physiological studies investigating the mechanisms of thirst and drinking. His curious and expansive nature led him into neurophysiological and neuroanatomical studies of the hypothalamus, under the tutelage of an early collaborator, John I. Johnson. In the early 1970s, Glenn began to apply these methods to studies of oxytocin and vasopressin cells in the supraoptic and paraventricular nucleus, and it was in the study of these magnocellular neurosecretory neurones that Glenn was to make seminal and lasting contributions, from over 150 peer-reviewed articles and many insightful reviews and chapters.

With Charles Tweedle, Glenn published landmark papers in 1976 and 1977 (1, 2) demonstrating a dynamic morphological relationship between glia and neurones, a relationship sensitive to the physiological state of the animal. This foray into quantitative electron microscopy served as foundation for a series of papers on neuro-glial relationships in both the hypothalamus and neurohypophysis, and crystallised Glenn's interest in how neurones and glia intercommunicated to shape their structure and function.

In 1977, Glenn took a sabbatical with Gary Lynch at the University of California, Irvine to learn the brain slice technique, and, soon after, his laboratory was the first to apply this technique to the hypothalamus (3). Glenn began collaborating with Ed Dudek, whose laboratory then made the first published intracellular recordings from neurosecretory neurones in the rat hypothalamic slice preparation and the first reports of dye coupling in these neurones. Glenn continued developing this technique on sabbatical with Bill Mason at the Babraham Institute in Cambridge in 1981, where, among other things, he studied the synaptic, cholinergic excitation of phasic neurones. This would be the first of two sabbaticals Glenn would spend in England, a country, and whose people, he greatly admired. On his second visit a few years later, he and John Bicknell demonstrated that vasopressin and adrenaline directly altered pituicyte morphology and calcium regulation. Fogarty and Guggenheim Foundation Fellowships supported Glenn's time in Cambridge, where he became a member of Corpus Christi College and a University of Cambridge Senior Scholar.

Electrophysiological studies characterised the Hatton laboratory until the end and, along the way, produced many revolutionary, sometimes controversial findings. A short list, in no way comprehensive, includes evidence that: (i) dye-coupling (suggestive of electrotonic communication) among neurosecretory neurones was prominent, modulated by transmitters, sex steroids and physiological state; (ii) phasic bursting activity characteristic of vasopressin cells occurred independently of synaptic transmission; (iii) the degree of intrinsic buffering by calbindin determined whether magnocellular neurones fired phasically or not; (iv) the slow depolarising afterpotential underlying phasic activity resulted from a calcium-

dependent decrease in a potassium current; (v) histamine released from tuberomammillary neurones mediated fast, H1-receptor-mediated excitatory postsynaptic potential (epsp) and fast, H2-receptor mediated inhibitory post synaptic potentials on supraoptic neurones; and (vi) acetylcholine mediated fast e.p.s.p.s via alpha-7 nicotinic receptors. Most recently, in a final and amazingly productive period, Wang and Glenn provided intriguing evidence that oxytocin promoted milk-ejection-like bursting in supraoptic neurones by way of complex, second messenger pathways involving prostaglandins, actin mobilisation and pituicyte remodelling.

Glenn's career had many marks of distinction. He was continuously funded from the National Institutes of Health, from which he was twice awarded the prestigious Jacob Javits Award as well as a Career Development Award. He served as president of the Association of Neuroscience Departments, on many National Institutes of Health and National Science Foundation panels, and on journal editorial boards. He was very proud to have edited, with Vlad Parpura, the remarkably successful book: *Glial-Neuronal Signaling* (4).

Glenn was an exuberant scientist who never lost the zest for discovery or debate. He was fearless in approach, unguarded in opinion. He was an outstanding and demanding teacher, a critical yet supportive colleague. As will be shown below, although the body of his work is large in scope, it remained focused on uncovering the neuronal mechanisms underlying vasopressin and oxytocin release.

Introduction

The story of oxytocin and vasopressin (5) began in 1895, when Oliver and Schafer reported that extracts of the posterior pituitary gland could affect urine flow, although what they described was a diuretic rather than an antidiuretic effect. In 1906, Dale reported that such extracts could also promote uterine contractions, and soon after, effects on mammary secretion were reported but confusion about the renal effects persisted. In 1917, it was reported that 'every text-book dealing with this matter states as an ascertained fact that extracts from the posterior lobe are diuretics' (6). Nevertheless, by the 1950s, it was clear that one of the factors present in extracts of the posterior pituitary gland must be 'the' antidiuretic hormone, and it had been proposed that another was a factor which caused milk let-down, and which was released as a consequence of afferent neural stimuli arising from the teats during suckling.

In 1950, Barry Cross, then a student of Geoffrey Harris, (7) showed that electrical stimulation of the pituitary neural stalk could elicit milk let-down in anaesthetised lactating rabbits and, in 1951, Harris (8) would write that 'Stimulation of the nipples by suckling appears to excite a reflex nervous release of oxytocin, and so contraction of the myo-epithelial cells'. However, he went on 'It is not yet beyond doubt that oxytocin is alone involved as the humoral stimulus to milk withdrawal. However, if this is so, and the above results are confirmed, then the oxytocic substance will have been raised to the status of an oxytocic hormone'. Confirmation soon came; relatively pure extracts of these factors were now available for physiological studies, and the structures of oxytocin and vasopressin were established (9, 10).

It was also becoming accepted that these factors were produced not in the pituitary but in the hypothalamus, and that they were packaged in membrane-bound granules, which were transported along axons. By 1951, Bargmann and Scharrer (11, 12) had shown both that dehydration caused these granules to disappear from the posterior pituitary, and that, when the axons of the hypothalamohypophysial tract were cut, these granules disappeared from the posterior pituitary but accumulated at the end of the neuronal stump. This led ‘doubters [to] belatedly and often reluctantly accept that neurohypophyseal hormones were indeed manufactured in the hypothalamus’, and the concept of neurosecretion had arrived (13). Then Douglas and Poisner (14) showed that these vesicles were released by calcium-dependent exocytosis and in their discussion, ‘attention is drawn to the similarities in the processes of “stimulus secretion coupling” in neurosecretory cells, chromaffin cells and neurones and it is suggested that a common calcium-dependent mechanism may be responsible for release of their various products’.

Thus, by the time that Glenn Hatton began studying the hypothalamo-neurohypophysial system, the stage was set. These neurones offered an opportunity not only for answering particular questions about posterior pituitary secretion, but also for establishing general principles of how the electrical activity of neurones relates to their physiological roles. Glenn’s first scientific paper, on water regulation in the rat, was published in 1965 (15) and his first anatomical paper, a study of the supraoptic nucleus, came in 1972 (16). This nucleus is remarkably compact and homogeneous; in the rat, virtually every supraoptic neurone projects directly to the posterior pituitary to secrete either vasopressin or oxytocin. To Glenn and many others, it was apparent that this offered unparalleled experimental opportunities. Even today, when we seek to understand the role of neuronal populations in the brain it is often as though ‘we see through a glass darkly’. We may perhaps know that these or those neurones do this or that in some or some other situation, but to know not only what neurones do, but also the physiological consequences that follow, and how those consequences come about, is usually obscure. However, the magnocellular neurones are output neurones of the brain, their activity is at the very end of the chain of information processing, their activity results directly in secretion of oxytocin and vasopressin, and the consequences of that secretion are either known or are amenable to study. Studies of these neurones therefore are deeply imbued with physiologically meaning.

For this reason, the supraoptic system became a model system of profound importance and, in the next 30 years, it yielded a succession of fundamental advances: it became a ‘Rosetta stone’ for neuroendocrinology and neuroscience (17). Each advance was greeted first by the assumption that this system, this bastard child of an unnatural coupling of brain and pituitary, was somehow peculiar; but then, slowly, came the creeping recognition that its peculiarities exemplified important principles of wide relevance. When Glenn started his work on this system, the oxytocin and vasopressin cells were the only peptide neurones in the brain; now we see the brain as an intricate mosaic of many populations of neurones that secrete a vast diversity of peptides (18).

In 1972, as Glenn was beginning his studies of the supraoptic nucleus, Jon Wakerley and Dennis Lincoln (19, 20) published their first observations on the milk-ejection reflex. They had used the new technique of antidromic identification to great effect. Subsequent to the

landmark studies of neurosecretory neurone physiology in fish (21), Kinji Yagi had shown that the axons of the magnocellular neurones in rat, when stimulated electrically, would conduct action potentials both orthodromically (i.e. towards the nerve endings) and antidromically (i.e. towards the cell bodies) (22). Earlier attempts to study hypothalamic neurones in mammals had relied on stereotaxic placement of electrodes and subsequent histological verification but, at best, this could only affirm the approximate site of recording, and not the identity of particular cells. However, using antidromic identification, neurones recorded from the vicinity of the supraoptic and paraventricular nuclei could be identified with certainty as magnocellular neurosecretory neurones if they could be activated antidromically by stimulation of the pituitary stalk. Wakerley and Lincoln could be sure that every cell that they identified must be either a vasopressin cell or an oxytocin cell, and soon they could take the next step: distinguishing one cell type from the other.

Wakerley and Lincoln found that, during suckling the oxytocin cells behaved in a way that was completely unanticipated (23) (these cells did not simply increase their electrical activity in response to suckling; far from it, their average activity scarcely increased at all). Instead their activity was re-organised: they fired in intermittent brief, high-frequency bursts, each lasting only a few seconds, and these bursts occurred synchronously in all the magnocellular oxytocin cells in both the supraoptic and paraventricular nuclei. The vasopressin cells had surprises too; many of them also showed bursts not in response to suckling, but in response to dehydration or haemorrhage. However, their bursts were much longer (tens of seconds), slower and, crucially, were not synchronised: a pattern known thereafter as phasic firing. This posed a host of challenges to the experimental community. What was so important about bursts? How did they arise, and why were they synchronised for oxytocin cells but not for vasopressin cells? And what was so special about suckling that this stimulus could evoke bursting in oxytocin cells when other ways of activating the cells did not? (24).

Phasic firing

Glenn had begun his career with studies of water regulation, and he turned to these homeostatic processes when examining the morphological and electrophysiological responses of supraoptic neurones to physiological challenge. In 1974, Walters and Hatton (25) reported the results from a study of the electrical activity of supraoptic neurones during 5 days of dehydration. The study is, in retrospect, flawed, in that the authors had not adopted the technique of antidromic identification. In relying on stereotaxic techniques, their results were diluted by the inevitability that an unknown number of their recordings were not from neurones that project to the pituitary but from neighbouring, non-neurosecretory neurones. Nonetheless, the outcome was striking: dehydration produced little change in the mean activity of cells except in those which showed phasically patterned activity; in these, dehydration produced a marked intensification of phasic bursting. This focussed Glenn's attention on the importance of phasic firing. 'It is tempting,' he said, 'to suggest that ... hormone depletion is more closely related to the activity within bursts in neurosecretory neurones' than to the level of activity *per se*.

This led Glenn to make phasic firing a focus of his studies. He had (correctly) inferred from morphological observations of the nucleus circularis (a small accessory nucleus of magnocellular neurones) that magnocellular neurones were directly osmosensitive, against the prevailing dogma that their osmosensitiveness was a passive response to osmoreceptors outside the hypothalamus (26). To test this, he saw the need for an experimental preparation in which the extracellular environment could be tightly controlled, and this led him to the *in vitro* hypothalamic slice preparation (Fig. 2) that he was to exploit throughout his subsequent career (3).

Working with Ed Dudek, Dave Andrew and Brian MacVicar in hypothalamic slices, Glenn collaborated on the first intracellular recordings of phasic firing (27, 28), and later was to show that phasic neurones were immunoreactive for vasopressin, but not oxytocin (29). He came to recognise that the key difference in the electrophysiological phenotypes of oxytocin and vasopressin cells was in how they handled intracellular Ca^{2+} (30), and he went on to show that the two cell types express different calcium binding proteins (31). He argued that the depolarising after potential (DAP), a key factor in phasic firing, mainly reflected a Ca^{2+} -dependent inhibition of outward K^{+} conductances, and proposed that the DAP, at depolarised potentials, was amplified by slow, Ca^{2+} -induced Ca^{2+} release, leading to a bistable plateau potential (32, 33) and hence phasic firing (34-36).

Significance of phasic firing

While Hatton and Walters were studying the effects of dehydration, Richard Dyball was studying the effects of giving rats 2% NaCl to drink for 3 days, and was using Yagi's method of antidromic identification to identify the magnocellular neurones (22). Dyball and Pountney (37) saw a similar intensification of phasic activity, but also saw an increase in the activity of nonphasic cells. This study was still confounded by the fact that many of their neurones were oxytocin cells not vasopressin cells and, in 1978, Wakerley *et al.* (38) sought to eliminate this uncertainty by looking at the effects of brief dehydration (up to 24 h) in lactating rats, where antidromically identified supraoptic neurones could be identified as vasopressin cells or oxytocin cells by their response to suckling. They too saw an intensification of phasic bursting in vasopressin cells, although the clearest change was an increase in the proportion of vasopressin cells that fired phasically, which happened within 6 h of dehydration. However, in this study, prolonged dehydration produced a much more dramatic increase in plasma osmotic pressure (an increase from 294 to 340 mOsm/kg at 24 h) than was seen by Hatton and Walters (39) (an increase from 293 to 302 mOsm/kg at 24 h), probably because of the greater fluid loss associated with milk production in lactating animals.

Taking all the results together, it would appear that, in severe acute dehydration, most vasopressin cells develop a phasic firing pattern; thus, most vasopressin cells have the capacity to fire phasically. In 2009, Scott *et al.* (40) returned to this question; they identified vasopressin cells by their responses to peripheral injections of cholecystokinin, a stimulus that excites oxytocin cells but inhibits vasopressin cells, and this let them determine how many of the vasopressin cells in dehydrated rats were actually firing phasically. After 24 h of

dehydration, they found intensified firing in phasic neurones, but no change in the proportion of vasopressin cells that fired phasically (40).

Thus, it appears that all vasopressin cells may be able to fire phasically but, in normal rats, even after prolonged osmotic activation, only some actually do so. Phasic bursting is much more efficient at causing vasopressin secretion than continuous firing (24), and so we can infer that cells that fire phasically will more rapidly deplete their stores of vasopressin than cells that are firing continuously (or slowly and irregularly). However, the secretion of vasopressin in response to electrical activity is proportional to the size of the releasable pool of hormone (41). Thus, we have a paradox; cells that develop a pattern of firing that is most efficient will, during chronic stimulation, lose that efficiency because they will be preferentially depleted of their content. Accordingly, is phasic firing really more efficient for secreting hormone in the long run?

There appear to be three possible answers. First, perhaps phasic firing is truly efficient only for acute secretion of vasopressin. Perhaps under chronic stimulation, those cells with inefficient patterns of activity become more important for overall vasopressin secretion because they have conserved their stores more parsimoniously. If so, heterogeneity in the behaviour of vasopressin cells is important for the ability of the vasopressin system to maintain secretion for prolonged periods.

Second, we cannot estimate the efficiency of stimulus–secretion coupling in chronic conditions without considering the effects of increased synthesis. Perhaps synthesis is activated more strongly in cells that secrete most? This would appear to require a link between either the pattern of electrical activity, or the state of the stores, and peptide expression. At present, we have a very poor understanding of how vasopressin synthesis is coupled to electrical activity; most likely that there is co-regulation, although whether patterns of activity that optimise secretion also optimise synthesis is not known.

Third, perhaps the electrophysiological phenotype of vasopressin cells is dynamically regulated during chronic dehydration, such that cells ‘cycle’ between periods of phasic firing, when they actively secrete hormone, and periods of relative quiescence when they recuperate, and rebuild their stores. Vasopressin cells intercommunicate by soma-dendritic release of several factors, including vasopressin itself, that act as ‘population feedbacks’ over varying spatial and temporal scales (42). These feedback factors may also be subject to activity-dependent depletion. Accordingly, an actively secreting cell may inhibit its neighbours, allowing them to build up their stores but, when it exhausts its secretory pool, those neighbours will be disinhibited, and will in turn become the active inhibitors (43).

Effects of chronic stimulation

Glenn saw that to understand the behaviour of vasopressin cells during chronic stimulation, it is not enough to study responses to acute stimulation; it is also important to understand how synthesis is controlled. Before the era of *in situ* hybridisation histochemistry, peptide synthesis could be studied only indirectly, and Hatton and Walters (39) set about looking for changes in nucleoli, intranuclear structures where ribosomal RNA synthesis, processing, and

ribosome subunit assembly, take place. They saw that, within even 2 h of dehydration, there was an increase in the number of supraoptic neurones that had multiple nucleoli, and the incidence of multiple nucleoli increased with progressive dehydration. The earliest change occurred before any measurable change in plasma osmotic pressure, suggesting that protein synthesis increases in supraoptic neurones well before any significant depletion of pituitary content, or any gross systemic imbalance. Hatton and Walters (39) speculated that this arose as a result of feedback of secreted hormone to the neurones; we now know that oxytocin and vasopressin do not cross the blood–brain barrier except in trace amounts, and would rather interpret this as evidence of coupling of electrical activity and synthesis.

Morphological plasticity in the supraoptic nucleus

Another observation from these studies caught Glenn's attention. During progressive dehydration, there was a remarkable hypertrophy of the supraoptic neurones: a doubling of the cross sectional area of the soma after 5 days of water deprivation, equivalent to a four-fold increase in soma volume and surface area (44). Glenn recognised that such a large change in cell volume meant that, during dehydration, there must inevitably be extensive structural reorganisation. In the first of a series of soon to be classic papers with Charlie Tweedle, he set about dissecting that reorganisation by studies at the ultrastructural level.

The first of these papers, published in 1976, included a study of the nucleus circularis, an accessory nucleus of magnocellular neurones that showed even greater changes in nucleoli and cell size than the supraoptic nucleus (1). The abstract includes the telling sentence: 'Water deprivation, even for 1 day, brought about a significant increase in both percentage of cells and membrane surface in contact [in the supraoptic nucleus and the nucleus circularis] *apparently by the retraction of fine glial processes from between the somata*'.

This observation raised many questions. What mechanisms were responsible for these changes, and what could their physiological significance be? In the neurohypophysis, Tweedle and Hatton found similar changes: 'Pituicyte processes retract from both areas when hormone demand is high, allowing the neuronal terminals direct access to the perivascular space'. They showed that osmotic challenges could produce similar changes *in vitro* in neurohypophysial explants (45).

They soon also showed that similar changes accompanied lactation and parturition; in late pregnant rats and in lactating rats, the extent of direct soma–somatic apposition between supraoptic neurones was significantly increased, indicating that the glial processes that normally surround the cell bodies had retracted. In addition, there was an increase in the number of 'double synapses' in the supraoptic nucleus, with instances of a single nerve ending making contact with each of two adjacent neurones (46–48). The dendrites of the magnocellular neurones also adapted and, in conditions of chronic stimulation, formed what he called 'dendritic bundles' (49), in which several dendrites directly apposed to each other were collectively wrapped in a glial 'overcoat'. Finally, more of the magnocellular neurones appeared to be in direct electrical communication with each other, apparently through gap junctions (50).

But what did these changes signify? During periods of little hormone secretion the neurosecretory axons are enclosed by the glial cells but are released under conditions of increased hormone demand, and from this, Glenn speculated that 'This may provide the morphological framework for pituicyte involvement in inhibition and facilitation of hormone release from the neurohypophysis'. Because these changes appeared to accompany several different physiological conditions, they were probably regulated neuronally, and because similar changes occurred in both the hypothalamus and the neurohypophysis, which contained only axons and pituicytes, they were probably regulated by the magnocellular neurones themselves. However, although the pituicytes in the neurohypophysis might be responsive to products secreted from the nerve endings of the magnocellular neurones as well as other neuromodulators (51, 52), there was no reason to believe that the hormones themselves were secreted within the hypothalamus – the axons, so far as was known, went only to the neurohypophysis, and there were few if any local projections within the magnocellular nuclei. With Bill Mason, Glenn set about looking for evidence of axon collaterals, and could show that some collaterals arose outside the nucleus (53), although he found no convincing evidence that any of them returned to terminate in the nucleus. Nevertheless, in 1986, these workers were able to show that both oxytocin and especially vasopressin could be released from the supraoptic nucleus *in vitro* (54). Although we now know that this release is predominantly from dendrites, such an understanding was still far in the future.

Glenn was not alone in these speculations; until 1982, his published studies had been confined to the effects of osmotic or volaemic stimuli. In 1981, Theodosios *et al.* (55) reported that a similar increase in soma-somatic apposition apparently attributable to glial cell retraction occurred during lactation, and this led them directly to the hypothesis that these ultrastructural changes might serve as morphological bases for synchronisation of electrical activity between the oxytocin cells. Importantly, they saw another key morphological change that accompanied the glial retraction: they reported an increase in the number of 'double synapses' in the supraoptic nucleus-synapses that contact each of two adjacent neurones (Fig. 3). They reported that, in lactation, these changes affected only the oxytocin cells. Similar changes occurred in response to dehydration but, again, specifically affecting the oxytocin cells. At the same time, Glenn was also studying the brains of lactating and late pregnant rats (46) and they also saw this striking increase in the number of double synapses.

Functional significance of morphological plasticity

Glenn was aware that the changes might well prove to be of passing interest. They might be epiphenomena: secondary consequences of the chronic hyperactivation of the neurosecretory cells, changes that perhaps were forced to physically accommodate the hypertrophy of the magnocellular neurones (56), or serving perhaps to sustain their increased metabolic demands. Although the morphological changes appeared likely to have functional consequences, it did not necessarily follow that they produced any changes in the behaviour of the magnocellular neurones. This might be considered paradoxical and is worthy of reflection. It is likely that the hypertrophy of magnocellular neuronal somata is an adaptation to allow increased synthesis of the hormones. However, all else being equal, an increase in

cell surface area will entail both a reduction in the density of afferent synapses, and changes in the passive electrical properties of the neurones. Accordingly, compensatory changes, especially perhaps in the number of synapses, might be required for the cells to retain their normal functionality, something suggested by Glenn's own work (56). The appearance of a new form of synapse, the double synapse, appeared to argue against this, although even this is not clear. A typical axon makes many thousands of synaptic contacts, and we might expect that any single axon that enters the supraoptic nucleus contacts many different supraoptic neurones; thus, so are double synapses really anything special? Perhaps double synapses arise more often simply because of the glial retraction, and perhaps they do not in fact reflect any increase in the sharing of afferent input.

These morphological changes were not confined to any single physiological state; they were observed during late pregnancy, when oxytocin cells first show synchronised bursting and throughout lactation, but also during chronic dehydration, to which oxytocin cells respond with greatly increased synthesis and secretion of oxytocin, but during which they do not display synchronous bursting. Although, in lactation, the changes were specific to oxytocin cells, Glenn concluded that the glial changes in dehydration affected both vasopressin cells and oxytocin cells (identified immunocytochemically) (57), and they occurred in the lateral, vasopressin- rich portion of the paraventricular nucleus as well as in the supraoptic nucleus (58). Similar glial changes occurred in the posterior pituitary affected both oxytocin nerve endings and vasopressin nerve endings (59). Thus, there was no clear indication that the changes had a functional significance that was specific either to one particular cell type, or to any particular physiological state, or any particular mode of behaviour, such as bursting.

Glenn noted that, in basal conditions, the neurohypophysial pituicytes occupy large portions of the basal lamina and engulfed axons and many nerve terminals. He first thought that the pituicytes might represent a diffusion barrier that limited hormone secretion in times of low demand (60). This suggestion encountered immediate objections: it is hard to see the utility of regulating the delivery to the blood of hormone that has already been secreted, and any explanation on these lines appeared unlikely to help our understanding of why similar changes occurred to the astrocytes in the supraoptic nucleus. However, the general idea that the pituicytes might exert some kind of restraining influence on secretion from the nerve terminals persisted. Perhaps a factor secreted from the pituicytes and astrocytes might influence neuronal excitability at both cell bodies and terminals.

However, the possibility that these morphological changes might have major implications for the functional behaviour of supraoptic neurones was too important to be overlooked.

Francoise Moos and Phillipe Richard showed compellingly that oxytocin itself was a key factor (61) and that, almost certainly, the bursting arose as a result of interactions amongst the oxytocin cells themselves (62). However, the bursting could not be observed in any *in vitro* preparation, so the opportunities for direct mechanistic dissection of the phenomenon were limited.

Glenn thus addressed the possibility that increases in cell-cell contact might increase communication between supraoptic neurones, and thereby serve a synchronising function, particularly in the oxytocin cells participating in the milk-ejection reflex. This

intercommunication might involve the proliferation of double synapses, also perhaps facilitated by the retraction of the thin glial processes from between the neurosecretory cell somata. However, Glenn focussed his attention on another possibility, that intercommunication was facilitated by an enhancement of electrical coupling through gap junctions, the appearance of which might be promoted by direct soma–somatic and dendro–dendritic apposition. This was a hypothesis that could be tested and, importantly, could be tested in the hypothalamic slice preparation.

Dye coupling between neurones

Glenn Hatton was involved in two seminal studies reporting that, when a supraoptic neurone recorded in a hypothalamic slice preparation was injected intracellularly with the fluorescent dye Lucifer Yellow, sometimes the dye would be found in several neurones (63, 64). They concluded that the dye must be passing between cells, probably through gap junctions, and that this must mean that the dye-coupled cells were also electrically coupled. Dye coupling was relatively uncommon in most experiments only one cell was labelled by dye injection but, in about one-third of cases, a few other cells (usually between one and three) were also labelled. Both oxytocin cells and vasopressin cells were coupled, although oxytocin cells were only coupled to other oxytocin cells and vasopressin cells to other vasopressin cells. Importantly, the incidence of dye coupling depended on the physiological status of the rats from which the hypothalamic slices were taken: there was more coupling in slices from dehydrated rats, and more when the slices were incubated in hyperosmotic medium (64). There was also more coupling between oxytocin cells in slices from lactating rats (65–67). The extent of dye coupling could also be modified by electrical stimulation of olfactory afferent inputs to the cells of the supraoptic nucleus (68), a projection that Glenn had been the first to describe. Interestingly, this effect was observed in lactating rats only. Dye coupling could also be enhanced by application of histamine, again an afferent projection system that Glenn had been the first to study (69). Finally, dye coupling was modified by gonadal steroids in both male (70) and female rats (71).

However, the physiological significance of dye coupling was far from clear; gap junctions could be observed in freeze fracture replicas of the supraoptic nucleus (SON), although these may have well been between astrocytes (63), and images of gap junctions between SON neurones had not been produced with transmission electron microscopy. Was dye-coupling therefore an artefact of the slice preparation, perhaps processes that are damaged in the cutting of a slice, reseal imperfectly or sometimes aberrantly, leading to the partial fusion of some cells? The fact that the incidence of dye coupling appeared to be physiologically regulated argue against this; but perhaps in hypertrophied cells, or when glial processes are retracted, aberrant cellular fusion might occur more often. The extent of dye coupling in the supraoptic nucleus was also much less than that observed in other areas of the nervous system, where electrical coupling was accepted as fact. Indeed, when testing for electrical coupling, only nine of 150 pairs were coupled, and these weakly (72). That oxytocin cells were coupled to other oxytocin cells was certainly suggestive but, because oxytocin cells and especially their dendrites are largely clustered together, this did not rule out the possibility that the coupling was an experimental artefact (e.g. from dye efflux along the pipette into neurones impaled in passage). Dye coupling was also as common among

vasopressin cells as among oxytocin cells, and this posed an obvious problem; although oxytocin cells in some circumstances show synchronised bursts, vasopressin cells are strikingly asynchronous in their activity. If only a minority of oxytocin cells are coupled to any other cells, how could this explain synchronisation throughout the population?

To all these arguments Glenn had counter-arguments: the slice preparation might underestimate the extent of coupling by severing dendrites (66) and the consequences of electrical coupling might vary depending on exactly where the coupling occurred, how strong the coupling was, and how electrical effects interacted with intrinsic mechanisms, as well as on the dye used (67). Glenn argued that extracellularly deposited dye never resulted in dye coupling in his studies, and that its regulation by so many factors made it unlikely to be an artefact. Nevertheless, most of Glenn's colleagues were cautiously sceptical, and few followed him down this path, even when he reported that expression of mRNA for connexin 32, a neuronal gap junction protein, was dramatically up-regulated in late pregnancy and parturition in the supraoptic nucleus (73).

Neuronal–glial functional interactions

Glial plasticity also had other far reaching implications that were initially raised by Glenn's work on taurine, an inhibitory transmitter localised in astrocytes (74) and in the pituicytes of the neurohypophysis (75, 76). Taurine is released from supraoptic astrocytes and pituicytes through hypo-osmotic efflux, and it inhibits magnocellular neurones or their terminals by actions on glycine receptors (77, 78). Thus, during hyperosmotic stimulation, a tonic inhibition would be lifted by two mechanisms: the direct hyperosmotic response of the glial cells to reduce taurine release, but also by removal of glial processes from the magnocellular targets.

Another consequence of glial process withdrawal involves the modulation of synaptic plasticity, where glial withdrawal during lactation allows freer glutamate diffusion and prolonged glutamate action on presynaptic metabotropic receptors as a result of the removal of glial-associated glutamate transporter uptake sites (79). This type of modulation is now considered to extend to the regulation of NMDA receptors and long-term potentiation in magnocellular neurones, through the glial-derived NMDA receptor co-agonist D-serine (80).

Inputs to the supraoptic nucleus

As covered above, much of Glenn's work was focussed on the substrate for intercommunication within the supraoptic nucleus, although the behaviour of these neurones *in vivo* depends on an interplay between intrinsic mechanisms and synaptic input, and Glenn was acutely aware that the character of particular neuronal inputs might be instrumental in determining key facets of the behaviour of supraoptic neurones. His work involved the study of four afferent systems in particular; one of which he identified as of likely significance to understanding the puzzle of the milk ejection reflex.

His first substantial contribution was in studies of the cholinergic input to the supraoptic nucleus. With Bill Mason, he showed that an area anterior to the supraoptic nucleus was rich

in cholinergic cells that provided a monosynaptic excitatory input to both oxytocin and vasopressin cells acting via nicotinic receptors (81, 82). He then showed that the tuberomammillary nucleus was the source of another direct projection, this time from histamine neurones. Again, both oxytocin and vasopressin cells were innervated monosynaptically, although with quite different consequences. Oxytocin cells were inhibited via activation of chloride channels, which could be blocked by histamine receptor subtype 2 (H2) antagonists, whereas vasopressin cells were depolarised via inhibition of a K^+ leakage current mediated by H1 receptors linked to GTP-binding proteins and Ca^{2+} -independent pathways (83). This provided evidence of the importance of second messenger pathways in transmitter effects. Histamine, working through H1 receptors, had previously been found to promote phasic bursting activity (84), probably through its ability to enhance DAPs (85) and by inducing direct membrane depolarisation (86). Glenn subsequently showed that this enhancement was mediated by phospholipase C and Ca^{2+} mobilisation from inositol 1,4,5-trisphosphate receptor-sensitive stores (87). Glenn was also alert to the possibility that the glial cells might be intermediate hosts of elements of some of these pathways. 'It is possible', he speculated, 'that histamine first acts at supraoptic cells (*perhaps both neuronal and non neuronal*) and induces the production of nitric oxide, which then diffuses to nearby neurones and modulates synaptic transmission by a postsynaptic mechanism' (88).

The third pathway that attracted Glenn's interest was a monosynaptic input from the mitral cells of the olfactory bulb; this input, he concluded, used glutamate as a transmitter and terminated mainly on the dendrites of magnocellular neurones of just the supraoptic nucleus; the paraventricular nucleus was apparently not involved. He noted that the dendrites of supraoptic neurones extended well beyond the conventionally accepted boundaries of the nucleus, coursing ventrolaterally along the surface of the periamygdaloid cortex. Using anterograde and retrograde tracing techniques, Smithson *et al.* (89) showed that there was an extensive projection from mitral cells of the accessory olfactory bulb to this dendritic projection of supraoptic neurones, suggesting a direct pathway by which olfactory stimuli arising from the vomeronasal organ might reach the nucleus. In electrophysiological studies, he showed that stimulation of the lateral olfactory tract activated both vasopressin and oxytocin cells (90, 91).

However, the input that perhaps most excited Glenn's interest was the noradrenergic input to the supraoptic nucleus from the A2 cell group of the nucleus tractus solitarius in the caudal brainstem. This pathway innervates oxytocin cells specifically, activating them via α_1 -adrenoceptors, and is important in the regulation of oxytocin secretion during both parturition (92) and lactation (93). When Jon Wakerley (94) reported that he and Hideo Negoro could occasionally evoke milk-ejection-like bursts in oxytocin cells in hypothalamic slices from lactating rats with the α_1 -adrenoceptor agonist phenylephrine, Glenn seized this opportunity. He found that, when hypothalamic slices were incubated in a low Ca^{2+} medium, the effects of phenylephrine were quite consistent, and bursts could be evoked in slices from virgin female and male rats as well as from lactating rats (95). Importantly, the burst-like behaviour was not simply a response to depolarisation induced by phenylephrine: in the continuous presence of phenylephrine, oxytocin cells exhibited occasional brief intense bursts whose profile matched closely the profile of milk-ejection bursts observed *in vivo*.

This close, quantitative match to *in vivo* observations convinced Glenn that he was observing essentially the same phenomenon as that occurring during suckling.

Accordingly, it now seemed to Glenn that, although the morphological changes in the supraoptic nucleus that he had seen in lactation might facilitate milk-ejection bursting, they could not be a prerequisite for it. Instead, the bursts must be a specific response of oxytocin cells to ‘the challenge of an appropriate neurochemical environment’. Clearly, this response also involved the dendritic release of oxytocin, and he reported that oxytocin could excite oxytocin cells *in vitro* at remarkably low concentrations (indeed, concentrations so far below the affinity at the oxytocin receptor as to cause some sceptical disquiet) (96).

The milk-ejection reflex

For Glenn, understanding the milk-ejection reflex was one of the greatest remaining challenges for oxytocin neurobiologists and, in the last phase of his career, he faced it with vigour, assembling all of the experience and understanding that he had accumulated. The physiological plastic changes in the oxytocin system during pregnancy and lactation should, he believed, ‘provide instructive clues’ to the process, but they alone could not explain the reflex. The withdrawal of astrocyte processes in the nuclei, increased neuronal direct membrane apposition, increased synaptic contact, especially double synapses, and the presence of gap junctions all might have some part to play in co-ordinating burst activity, although many of these changes also affected vasopressin cells, and were seen in the oxytocin system in physiological circumstances that were not associated with bursting. However, now he had seen bursts in oxytocin cells in male rats following acute *in vitro* manipulations. His meticulous quantitative comparisons of these bursts with suckling-induced milk-ejection bursts led him to conclude that these bursts arose through essentially similar underlying mechanisms, comprising mechanisms that were intrinsic to the oxytocin cells and their partner astrocytes and dependent on oxytocin release from the dendrites.

Glenn concluded that the key must lie in the intracellular pathways that were activated by a particular chemical messenger released during suckling, and his last leap of imagination was to conceive that this might be linked to a rapid morphological reorganisation at a subcellular level that preceded the overt global morphological reorganisation of the nucleus. In his last papers (with Yu Feng Wang) Glenn provided the first evidence that oxytocin increases the formation of filamentous F-actin (F-actin) in oxytocin cells through prostaglandins (97, 98). F-actin is a key part of the dendritic cytoskeleton; it forms a matrix that can guide or constrain the disposition of oxytocin vesicles in the dendrites (99). Thus, the probability is high that dynamic F-actin reorganisation is a crucial link in oxytocin-prostaglandin-mediated excitation that may be a key link for oxytocin-evoked bursts (96). Prostaglandin E₂ can extensively activate neurones, as indicated by enhanced expression of *c-fos* mRNA (100), and has both pre- and postsynaptic excitatory actions on supraoptic neurones (101).

Oxytocin secretion from axon terminals in the neurohypophysis depends upon Ca²⁺ entry through voltage-gated channels evoked by action potentials. This secretion is highly nonlinear; clusters of action potentials result in summation of Ca²⁺ influx that potentiates the efficacy of action potentials. Only vesicles close to the plasma membrane can be released

in this way and, as these ‘readily releasable’ pools are depleted, the efficacy of action potentials declines until these pools can be replenished. By contrast, in dendrites, the coupling between activity and oxytocin release is much more complex. Action potential activity causes relatively little release, and high frequency discharge is even more important than in the terminals. However, dendritic peptide release can be evoked in an activity-independent manner by agents that act via mobilisation of the Ca^{2+} stores of the endoplasmic reticulum. Importantly, some second messengers can enhance the efficiency of action potentials in eliciting dendritic oxytocin release by a process called ‘priming’ (18, 102). Such messengers increase the pool of vesicles close to the plasma membrane that are available for release in response to voltage-gated Ca^{2+} entry, and, as mentioned above, the underlying mechanism involves reorganisation of the F-actin network (99).

Thus excitation of oxytocin cells through prostaglandins may be a key link for oxytocin-evoked bursts (96), and morphological reorganisation at the subcellular level (reorganisation of the cytoskeleton and priming) may be key to intercommunication between oxytocin cells, facilitated by morphological reorganisation at the cellular level (double synapses, gap junctions and dendritic bundling). These ideas are similar to those implemented in a computational model of the milk-ejection reflex by Rossoni *et al.* (103) that provides a close quantitative match to milk-ejection burst activity, and also provides a close agreement to qualitative features of the reflex in many experimental and physiological circumstances. Such a model cannot be taken as proof that the reflex works in this way, but it does demonstrate that this offers a feasible and apparently comprehensive explanation. It is too early to judge that we have a final explanation of the milk-ejection reflex but it looks as though we have a candidate.

There remain many uncertainties. Although Glenn showed that oxytocin can have strong excitatory actions on oxytocin cells at low doses, it is far from certain that oxytocin is the only communicator of excitation between oxytocin cells. Intriguingly, he and his colleagues showed that oxytocin cells (and vasopressin cells) express vesicular glutamate transporters, suggesting that magnocellular neuroendocrine somata and dendrites may be capable of glutamatergic transmission, possibly via small clear vesicles long known to be present in the magnocellular neurones but whose function has remained undetermined (104).

Last words

In writing about the legacy of a friend and colleague, we are conscious of saying too little to do justice to a treasury of science and wisdom, and yet of saying too much, that the man may be lost among the words.

In *Mother Tongue*, Bill Bryson quotes advice given by a car rental firm in Tokyo: ‘When a passenger of the foot heave in sight, tootle the horn. Tootle at him melodiously at first, but if he still obstructs your passage, then tootle him with vigour’. Glenn Hatton first played the bugle as a founder member of the Cavaliers Drum & Bugle Corps, formed in 1948 from the Chicago Logan Square Boy Scout Troop 111. His vigorous tootling took different forms throughout his life, and will be sorely missed. Fortunately, his score is there for all to read in a copious literature.

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Fig. 1.
Glenn I. Hatton (1934–2009). Reflecting on water balance. Photograph courtesy of William Hatton.

Fig. 2.

Hypothalamic slices suspended on nylon net in a recording chamber, as seen through a dissecting microscope. The chamber is backlit using a light pipe. Top: several coronal slices (300 μm) through the hypothalamus are visible. The size of the optic chiasm/tract is useful for distinguishing the serial order of the slices. Inset: oscilloscope record of a phasic bursting neurone typical in pattern to vasopressin cells recorded *in vivo*. This neurone was recorded from an accessory portion of the supraoptic nucleus. Bottom: a slice at higher magnification in which translucent nuclei appear against the opaque myelinated fibre tracts. AC, anterior

commissure; Fx, fornix; NC, nucleus circularis; OC, optic chiasm; OT, optic tract; PVL, lateral magnocellular paraventricular nucleus; SCN, suprachiasmatic nucleus; SOa, supraoptic nucleus, anterior portion. Modified with permission (3).

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Fig. 3.

Dynamic morphology of the supraoptic nucleus. Top: vasopressin and oxytocin cells (c) in the supraoptic nucleus are normally separated by the processes of glial cells (g). During dehydration, these cells hypertrophy and glial cell processes retract, leaving the somata and dendrites in close apposition. Glenn's observations led him to conclude that these close appositions involved increased coupling between neurones via gap junctions (green). He also recognised that oxytocin cells especially were increasingly innervated by 'double synapses' (red arrow), comprising synapses that contacted each of a pair of adjacent neurones. Bottom left: a double synapse. Reproduced with permission (47). Bottom right: increased dendro–

dendritic appositions giving rise to ‘dendritic bundles’ (three dendrites labelled “1” are directly apposed to each other). Reproduced with permission (105).

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