A STUDY OF THE SYNAPSE
Neuroplasticity and ATP Transmission

A personal account of the research of Professor Maxwell Bennett AO
1960-2020
Frontispiece. A nerve terminal, that releases ATP in smooth muscle, showing the rise and fall of calcium following a stimulus to the nerve at 0 time and followed for 3.5 sec. This is the first time a synaptic terminal has been shown functioning in its normal environment. For details see the legend to Figure 4 in Part II.
CONTENTS

Contents .................................................................................................................. 5

Preface .................................................................................................................... 7

Note to the Reader ................................................................................................. 9

Part I. Neuroplasticity of Synapses

1. The formation of synapses in embryonic development and following nerve injury .......................................................... 11

2. The plasticity of synaptic terminals in the release of transmitter substances ................................................................. 19

3. The retraction of nerves from their synaptic targets during development of the brain, the death of their cell bodies and neuron growth factors ........................................................................ 29

4. The retraction of nerves from their synaptic targets on dendrites following stress, the collapse of dendrites and the loss of grey matter ........................................................................... 37

5. The loss of functioning inhibitory synapses on neurons in the brain in schizophrenia ......................................................... 45

References .............................................................................................................. 53

Summary of Discoveries on Neuroplasticity ......................................................... 57

Research Papers on Neuroplasticity ................................................................. 63
(Complete set)
Part II ATP Transmission at Synapses

1. The electrical signs of inhibitory transmission to smooth muscles, and the identity of a novel transmitter substance, ATP .......................................................... 73

2. The electrical signs of excitatory transmission to smooth muscles, and the release of the transmitter substance ATP onto receptor clusters ............................................................ 79

3. Transmission to smooth muscles forming an electrical syncytium utilizing the novel transmitter ATP ..................... 87

4. Transmission between astrocytes forming an electrical syncytium utilizing the novel transmitter ATP ............ 93

References ........................................................................................................ 101

Summary of Discoveries on ATP Transmission at Synapses ... 105

Research Papers on ATP Transmission at Synapses .......... 109
(Complete set)
PREFACE

As a young teenager, I became interested in philosophy, especially that of A.N Whitehead and subsequently Ludwig Wittgenstein, whose work I was later to use in trying to understand the neural basis of our psychological attributes such as remembering and thinking (e.g. *Philosophical Foundations of Neuroscience* co-authored with Peter Hacker). I determined then to study these great philosophers of the first half of the 20th century at the university of Melbourne, my home town, but was prevented by doing so by my father who had decreed that I do electrical engineering and physics. This has been recounted, in my *Search for Knowledge and Understanding*. However while carrying out the edicts of my father I founded a group called The Athenian Society, consisting of like-minded undergraduates from a variety of disciplines whose aim was to present papers each week during term on great early 20th century philosophers. What emerged from the first year of these meetings was a consensus that the origins of consciousness constituted a central issue, one which I have held ever since, as manifest in the books I have authored or co-authored on this subject (see [https://www.amazon.com/M.-R.-Bennett/e/B001JS32MU](https://www.amazon.com/M.-R.-Bennett/e/B001JS32MU)).

However, on graduating I did not enrol in philosophy but in zoology, a subject in which one could study the nervous system and at the same time pursue philosophy as it impacts on the question of the relationship between the brain and the mind. On enrolling for a PhD I took up the task of examining how synapses work in the autonomic nervous system, that which controls the internal organs and is closely associated with our emotions. In particular I tackled the task concerning how the nerves in this system control the smooth muscles they impinge on at the neuromuscular synapse. It turned out that I was particularly adept at inserting tiny electrodes into these small muscle cells, about four one thousandth of a millimetre in diameter, so I could record the electrical signs of the release of substances onto the muscle cell from the impinging nerves. There quickly followed a series of revolutionary discoveries: that some of these nerves released substances that had not been identified before, leading to the identity of Adenosine Triphosphate (ATP) as a principal transmitter at nerve endings by my fellow PhD student Graeme Campbell, with considerable clinical implications for diseases such as thrombosis (Bennett, 2013); that the synaptic potentials propagated between the muscle cells which acted as an electrical syncytium in transmission and that in addition the transmitter substance was shown on theoretical grounds to diffuse from its point of release to produce a further integrated action. This work, among other
observations, was recognized by the premier physiological society in the world asking me to write a monograph on the work, while I was in my twenties, the first Australian to be invited to write for them (Bennett, 1972). During the final years of my PhD and the year I took to write this work I pondered on what area I should research next. To that end I first had discussions with Sir John Eccles in the Australian National University, who had won the Nobel Prize for his research on synapses in the brain. I then sought advice from Professors Peter Bishop and Liam Burke in Sydney University who had trained with Sir Bernard Katz in London, another Nobel Prize winner on the synapse but in this case on muscle. In addition I met with two philosophers in Melbourne, namely David Armstrong and Cameron Jackson. These discussions led to the conclusion that a totally new avenue of inquiry on the synapse was needed, that a phenomenon like memory must involve long-term changes at synapses, the making of new synaptic connections and perhaps the loss of others, what is now called the neuroplasticity of synapses. To this end I accepted the offer of a lectureship from Liam Burke in Sydney in 1968, where I could set up a laboratory to study neuroplasticity of the synapse. The following is an account of how young research students and I set about establishing this concept of synaptic plasticity, of moving the accepted tradition that synaptic connections are fixed genetically during embryonic development and neural networks ‘hard-wired’, to one of plasticity in which new networks are forming and others discarded throughout life.

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NOTE TO THE READER

I have selected a number of projects which my colleagues and I have worked on in the past 60 years that seem particularly worth highlighting because of the insights they have provided on the workings of synapses. Insufficient space has precluded me from mentioning all of those without whose support the research would not have been brought to a successful conclusion. Suffice is to say that the names of many of them appear in the extensive reference lists. But those who are not explicitly mentioned are the technical support staff without whose commitment the research would not have occurred at all. To those I am entirely indebted.
1. The formation of synapses in embryonic development and following nerve injury

This study began after my reading of the two-volume work of the early 20th century histologist Ramón y Cajal and particularly his student Jorge Tello, who had attempted with the techniques then available to establish how that most accessible of all synapses, occurring on muscle cells, matured during development. Also of great interest was the relatively recent discoveries in the 1960s of David Hubel and Torsten Wiesel concerning the waning and strengthening of projections of nerves to the developing visual cortex, possibly due to the loss or strengthening of the synapses there, although the complexity of the visual cortex prevented them from showing this. I decided that, given these previous investigations over half a century, what was first needed was to determine how synapses form initially during embryogenesis. With a student Alan Pettigrew, I set out to give the first description of the earliest synapse formation on muscle during development of the embryo (Bennett and Pettigrew, 1974). The muscle at this stage is just forming from the alignment of round cells into tubes, the myotubes, that will become muscle cells in the embryo, whilst the nerves grow into this cellular complex as single axons each ending in bulbous growth cones which then abut on the myotubes and cease growing (Figure 1). This is the first synapse. It is subsequently joined by other growth cones so a myotube receives several synaptic connections, and as the myotube matures to a muscle cell by growing only in length at each of its ends, these synapses become marooned in the middle of the muscle, so that at birth single muscle cells possess several synapses (Redfern 1970). Two questions are of great interest here: first, what seems to constrain the subsequent growth cones to only form synapses at what has become the middle of the muscle? Second, as it is known that mature muscles only possess a single synapse, when are the excess synapses eliminated and what determines which of them is to be eliminated? These are clearly critical questions of importance in the wiring of the brain, which was subsequently shown to possess neurons with excess numbers of synapses on them in development as do muscle cells; the claim made for the excess synapses in the brain is that their elimination leads to more accurate network connections (Katz & Schatz, 1996). Figure 2 shows that the excess synapses are eliminated in rodents over a period of about two weeks after birth, but what determines which ones are eliminated is still largely unknown, although there are now major insights into why the additional synapses are constrained to the initially formed synaptic site.
These insights have come largely from experiments on the synapses of mature muscle cells and are summarized in the diagrams of Figure 3. The mature distribution of synapses, one per muscle cell, are shown located in the middle of the cells (A), and this pattern is recapitulated if the muscle is deprived of its nerve by cutting it and then allowing the nerve to regrow from any position into the muscle as shown in B; so there is something special about the membrane of the cells at the middle of their length which triggers the nerve to form a synapse there. This property of the original synaptic site on the muscle cell is emphasized by the fact that attempts to produce additional synapses in a normally innervated muscle by introducing a nerve from another fails to do so, as shown in C. Furthermore, if an attempt is made to innervate a muscle deprived of its normal nerve by a distinctly foreign nerve it will almost completely fail to do so, as shown in D; the synaptic sites on the muscle cells not only determine where a nerve may form a synapse but also what kind of nerve can do so (Bennett and Pettigrew, 1976). All these observations could be explained by means of a mathematical model, called the Dual Constraint model, which postulated recognition molecules at the synaptic site on the muscle cell which could trigger a nerve terminal to form a synapse there, this site having been determined by the first growth cones to reach the muscle during development (Bennett and Robinson, 1989). Since that time an intense effort has been made to identify these recognition molecules, of which the leading candidates are MuSk (Burden et al., 2013) and Agrin (Tezuka et al., 2014), with those at synapses in the brain also being sought.

Figure 1 Distribution of nerves in the rat hemidiaphragm at 15 days gestation. A, the primary (phrenic) nerve (p) enters near the centre of the hemidiaphragm and divides into two secondary nerve branches (s) which traverse the myotubes (my) at right angles and extend to the pools of myoblasts (mb) at the ends of the hemidiaphragm. Smaller tertiary nerve bundles (t) leave the secondary nerve trunks and run in general parallel to the myotubes and obliquely into the pools of myoblasts. The tertiary nerve branches extend almost the entire length of those myotubes which are adjacent to the pool of myoblasts. Calibration, 100 um. b, tertiary nerve bundles at the end of the secondary nerve trunk radiating into the pool of myoblasts (mb) at the end of the hemidiaphragm. Calibration, 50 um. c, single axons leaving the tertiary nerve branches at a position 1 mm from the point of nerve entry into the hemidiaphragm and growing out between and along the myotubes (my) in this region. The tertiary nerve bundles arise from the secondary trunk (s) at the bottom of the plate. Some axons have been retouched since they pass out of the plane of focus of the three photomicrographs use to make up the plate. Calibration, 30 um. (From Bennett and Pettigrew, 1974).
**Figure 2** Changes with age in the mean number of terminals per muscle cell of the rat hemidiaphragm. Error bars represent the S.E. of the mean (n > 14). Bennett and Pettigrew (1974).
Figure 3 Diagrammatic representation of the pattern of innervation by fast (twitch) motor neurons of control muscles (A) and of operated muscles where there is a good (B) or poor (D) matching between the innervating axons and the muscle (after combined AgCgE staining of muscle sections used in this study). In each diagram, the myotubes are orientated vertically, and the small round dark spots represent the sites of the synapses in the muscle. Dashed lines represent portions of small blood vessels within the muscle.

(A) Control muscle. The nerve enters the muscle near the end-plate zone and after reaching the end plates, divides into two branches (each of 30 to 40 axons) which traverse the muscle at right angles to the longitudinal axis of the myofibers; these branches then give rise to small groups of axons (2 to 5 axons), each of which directly innervates the synaptic-sites at the end-plate zone.

(B) Reinnervated muscle or cross-reinnervated muscle where there is good matching between the motoneuron type and the muscle. When the nerve enters the muscle away from and proximal to the normal point of nerve entry, it divides into nerve trunks (<15 axons) which eventually reach the original end-plate zone by following blood vessels and the surface of myofibres down the long axis of the muscle. These trunks then divide into small bundles (2-3 axons) which can be observed throughout the end-plate zone, and which finally give rise to single axons that innervate the denervated synaptic sites ‘en passage’ or by collateral sprouting. Single axons can sometimes be observed coursing along blood vessels and myofibers, and occasionally ectopic synapses are formed near the point of nerve entry if the nerve is implanted into the muscle. No axons can be observed in the distal half of the muscle.

(C) Attempted hyperinnervation of muscle. When the additional nerve enters the muscle, it breaks up into small nerve bundles (3-5 axons) and single axons, which grow along blood vessels, connective tissue structures and sometimes along myofibers; the axons can be observed throughout the muscles, but no synapses are formed by these axons except occasionally near the point of nerve implantation. The innervation of the end-plate zone is as described for the control muscle.

(D) Cross-reinnervated muscle where there is poor matching between motor neurons and the muscle. As in C, the foreign nerve breaks up into small nerve bundles and single axons at its point of nerve entry into the muscle, and these spread throughout the muscle without any preferred orientation by following blood vessels and connective tissue elements. Denervated synaptic sites in the original endplate zone (small dots) are only rarely innervated, and very occasionally synapses are formed at the site of nerve implantation. (from Bennett and Pettigrew, 1976).
2. The plasticity of synaptic terminals in the release of transmitter substances

Nerve terminals at synapses release substances that in most cases chemically excite the cell they contact, be it a muscle cell or a neuron. It had long been held that all the terminals of a nerve released this substance with the same probability, which is fixed in maturity (Katz, 1969), but two PhD students Nick Lavidis and Greg MacLeod and I showed that this is not the case. This was made technically possible by the then recent discovery of a substance, DiOC$_2$(5), that allowed the staining and fluorescent visualization of nerve terminals on muscle cells (Figure 4, lower panels). These nerve terminals consist of branches of hundreds of individual synapses crowded together to form the branch, allowing one to visually guide a fine tipped recording electrode to synapses of choice along the tube (numbered in Figure 4). When the probability of transmitter release was recorded at the different synapses it was found to vary, declining markedly for synapses in the more distal sites along the branch of synapses (Figure 4, upper panels). It will be noted in this Figure 4 that in some cases the middle length of the terminal branch shows little change in probability, a feature that has been attributed to the distribution of sodium channels determining the duration of the action potential responsible for transmitter release (Ginebaugh et al., 2020). Another technical innovation, the use of fluorescent dyes such as FM1-43 that is taken up by live synapses in proportion to their release activity, was used to show that the probability of release changed for the more distal synapses along the length of a branch whilst the distance between synapses remained constant (Figure 5). This was confirmed by placing a triangulation of recording electrodes around synapses, so isolating the electrical signs of transmitter release from single synapses, and showing that this varied in accordance with the FM1-43 staining results (MacLeod et al., 1999). The question then arose as to what is the basis of this variability in transmission, and the surprising answer was that it is due to the plasticity of the synapses. High resolution recording of these over hours showed that new synapses form and others regress over relatively short time periods, and it is this neuroplasticity that introduces the variation in transmitter release at the very end of terminals (Figures 6 & 7). This work showed for the first time that even adult terminals can be under a continual state of remodelling.
Figure 4 Changes in $m_c$ (quantal content) along the length of medium-size terminal branches 981-120 um) at simple end-plates with two branches (A) and complex end-plates with several branches (B). In each case a drawing of the extent of the nerve terminal branching is given, derived from the DiOC$_2$(5)-stained nerve and cholinesterase-stained post-synaptic membrane. The numbers on the terminal give the sequence in which the recordings were made; arrows indicate the position of the micro-electrodes. (From Bennett et al., 1986).
**Figure 5** The intensity of FM1-43 staining along the entire length of terminal branches of motor-nerve terminals. A: simple terminal branch, about 40 um long, stained with FM2-43. P and D indicate the most proximal and most distal points of the branch, respectively. B: profile of the intensity of the FM1-43 staining along the branch at intervals of 0.13 um. C: average amount of FM1-43 fluorescence per blob in each one-tenth of the terminal length along the branch in A. D: average distance between the midpoint of the blobs in each one-tenth of the terminal length for the branch in A. E: complex of what is perhaps two parallel terminal branches, probably in the same synaptic gutter, stained with FM1-43. P and D are defined as in A. F-H: same as B-D, respectively; no allowance has been made for the fact that there may be two terminal branches present. Calibration in A is given by the abscissa in B and the abscissa in F gives the scale for E.

(from MacLeod, Gan and Bennett, 1999).
**Figure 6.** Individual clusters of synaptic vesicles, indicated by FM1-43 blobs, can appear or disappear from the ends of terminal branches over hours. FM1-43 blobs are shown along the distal portions of six terminal branches (A-F) after an initial staining with FM1-43 (left panel) and the same terminal branches are shown after restaining 16 hrs later (right panel). A-C show the appearance of new clusters of FM1-43 stained vesicles on these terminal branches (asterisk), whereas D-F show the disappearance of such clusters over the same period (asterisk). The scale bar shown in A is the same for all images. (from Macleod, Dickens and Bennett, 2001).
**Figure 7.** The growth of motor nerve terminal processes with respect to Terminal Schwann Cell (TSC) processes. Shown are the changes in terminal processes \((a, b)\), as well as their associated TSC processes \((c, d)\) over a time interval of 19 min in \(A\) and 52 min in \(B\). In each case, \(e\) and \(f\) give the superimposed images. In \(A\), a small terminal process appears between \(a\) and \(b\), and this follows the left-hand TSC process that has remained stationary over this time, as shown by \(c\) and \(d\). In \(B\), a small terminal process appears between \(a\) and \(b\), and this follows the central TSC process that shows little change over the time between \(c\) and \(d\). In \(A\), OG-5N was injected into the nerve terminal and AF568 into the TSC. In \(B\), AF488 was injected into the nerve terminal and AF568 into the TSC. Time stamps represent the time since capturing the first images of the nerve and TSC in the before–after series. The first images of filled TSCs were captured 5 hr 16 min \((Ac)\) and 3 hr 43 min \((Bc)\), respectively, after toads were killed.(from Macleod, Dickens and Bennett, 2001).
3 The retraction of nerves from their synaptic targets during development of the brain, the death of their cell bodies and neuron growth factors

The possibility that over half the cell bodies from which nerves emerge die during early development, as a consequence of their nerves failing to obtain a necessary growth factor from their targets on which they form synapses, had been shown to be very likely for neurons outside the brain by the Nobel Prize winner Rita-Levi Montalcini. But this had not been shown to be the case for the brain, although it had for the spinal cord (Hamburger, 1975). Bennett suggested to his colleague, the neuroscientist Bogdan Dreher, that this could be tested by observing during development the relatively accessible neurons in the retina, an out-pocketing of the brain. They were able to do this with a research student, Rebecca Potts (Potts et al., 1982) by using the then new technique of injecting a dye (HRP) into the area of the developing brain in which the retinal neurons form synapses; from there this particular dye can be transported by the retinal nerves back into their neurons in the retina, allowing them to be counted. When this was done immediately after birth it was found that about a 30% loss of neurons occurred within a few days (Figure 8). Accompanying this was a similar loss of nerves projecting to the brain as determined by electron microscopy, indicating that that these had lost their synaptic connections and degenerated along with their neurons in the retina (Figure 9; Lam et al., 1982). This was the first quantitative account of normal cell death in the developing brain.

We then attempted to identify the growth factor in the brain which is normally made available to nerves so as to keep their neurons alive according to the paradigm laid down by Levi-Montalcini for the nervous system outside the brain. To this end we used a unique florescent label for the developing retinal neurons of interest which allowed them to be identified on dissociating the whole retina into its constituent cells in a petri dish. These neurons were shown to die within 24 hrs when isolated in this way but they could be maintained viable if homogenates of the regions of the brain to which they normally project to form synapses was added to the dish. I then recruited a research student Mark Schulz and a biochemist Ralston to join us in order to determine the molecular species in the homogenate that was responsible for this growth factor effect (Figure 10: Schulz et al., 1990). It was a chondroitin sulphate proteoglycan, a very large molecule which was subsequently shown by another research student, Ms Huxlin, to maintain the viability of ganglion cells in vivo (Huxlin et al., 1995). So neurons die in the normally developing
brain, possibly as a consequence of their nerves failing to form stable synapses necessary for them to obtain a growth factor (Johnson et al., 1986).

**Figure 8.** Estimated number of cells containing HRP reaction products in retinal wholemounts of rats of different ages. The ages indicated on the abscissa refer the ages of the animals at the time of the perfusion. The block dots indicate the estimated number of labelled cells in the animals in which HRP had been injected 15-20 hrs before the perfusion. The triangles indicate the estimated numbers of labelled cells in the retinae of animals in which HRP was injected 12 hrs before birth. Finally, the open circles indicate the estimated numbers of labelled cells in the retinae contralateral to the ablated (within 12 hrs of birth) superior colliculus. HRP was injected in these animals 15-24 hrs before the perfusion. Inset: micrographs of the HRP-labeled cells in the whole mounted retina of 8-day old rat. HRP was injected on day one (5 hrs after birth of the animal). The picture was taken under a 100 x oil immersion objective. The photographed area is located about 1 mm from the periphery of the retina. Calibration bar = 10 um. Note that a number of cells located in the ganglion cell layer do not contain HRP reaction products. (from Dreher, Potts and Bennett, 1983).
**Figure 9**, Electron micrographs of optic nerves of rats of 3 different ages. Calibration bar is 1 um in A (upper), B (middle) and C (lower). A: optic nerve of newborn rat (postnatal day one). Note that the nerves are small, and all are unmyelinated. B: optic nerve of rat on postnatal day 6. At this age the number of fibers has fallen to adult values and myelination is just commencing (arrowhead). C: adult optic nerve, fully myelinated (Lam, Sefton and Bennett, 1982).
Figure 10. Elution profiles of Mono Q anion–exchange chromatography after enzyme treatment. A: Untreated. B: Treated with heparitinase. C: Treated with chondroitinase ABC. Solid line represents absorbance at 254 nm and the histograms the trophic units present. The trophic units present in A and B, fraction2, could be determined; no other fraction could maintain retinal ganglion cells above control levels. (From Schultz, et al., 1990).
4. The retraction of nerves from their synaptic targets on dendrites following stress, the collapse of dendrites and the loss of grey matter

About 66% of the volume of grey matter of the brain is taken up by neurons and their processes (Figure 11). If animals, including humans, are subjected to stress then it is known that certain regions of the brain loose substantial amounts of grey matter, as measured using non-invasive brain – imaging techniques such as Magnetic Resonance Imaging. For example, in the area of the brain involved in memory, called the hippocampus, there is a 10% decrease in volume (Figure 12B; Kassem et al., 2013). My colleagues and I showed in animals subjected to small and intermittent stress, such as in the case of rodents keeping them in the cylinder of a toilet role for 20 minutes each day for a few weeks, that a decrease in the synapses formed by nerves on the dendritic processes of neurons occurred (Figure 13). This in turn led to the shrinkage of these dendritic processes (Figure 13 B). We showed that dendritic processes make up 26% of the volume of grey matter (Figure 11), and that it is the loss of the dendrites that is responsible for the loss of grey matter. There is then a one-to-one relation between the loss of grey matter in different parts of the brain and the loss of dendrites there, as a consequence of synapse loss (Figure 12). This was the first study to establish that grey matter loss due to a mental condition arises as a consequence of the retraction of nerves that normally form synapses. It therefore pin-pointed the importance of finding how to prevent this regression of synapses following stress.
Figure 11 Volume of grey matter taken up by different volumes of its cellular constituents and their processes. Percentages are determined from the review of existing literature as calculated in Table 5 in Kassem et al., 2013.
Figure 12. In stressed animals, there is a linear relationship between the loss of Grey Matter Volume determined with high resolution Magnetic Resonance Imaging and the cumulative loss of dendritic volume such that the former can mostly account for the latter. (from Kassem et al., 2013).
**Figure 13.** Significant decreases occur in stressed animals in the cumulative length of apical dendrites of neurons, that is the sum of all different order dendrites in the Anterior Cingulate Cortex and CA1 hippocampus. a, photomicrograph of Golgi-stained pyramidal CA1 neurons of a non-stressed mouse. b, dendrites (at the same magnification as in (a) of a pyramidal CA1 neuron of a stressed mouse with shorter cumulative dendritic length (from Kassem et al., 2013).
5. The loss of functioning inhibitory synapses on neurons in the brain in schizophrenia

In order to be able to determine the function of synapses in the brain of those suffering from a mental disorder, one has to first develop a quantitative relation between synaptic activity in a brain area and a quantity that could be measured in that area with non-invasive imaging. I suggested to colleagues in the Yale Centre for Imaging that the energy expenditure of neurons in an area of interest due to synaptic activity should be quantitatively related to synaptic activity as measured with electrodes in the neurons. I contacted the Yale group because they were experts in measuring energy expenditure in the brain by following the utilization of radioactive glucose with Positron Emission Tomography (PET). The Table in Figure 14 showed this suggestion to be true: the bottom two rows illustrate the agreement between the measured expenditure of energy and that calculated from the synaptic activity, under a variety of conditions such as awake (AWK) and asleep (SLP) (Hyder et al., 2013). The quantitative relation between these quantities could be expressed as:

\[ E_{\text{tot}} = E_s + E_{ns} = \sum Ps(f) + \sum Pns \ n \]

where the energy providing molecule ATP used for signalling (Ps) and for glial non-signalling (Pns) are multiplied respectively by (f) the average cortical firing rate and by (n) the cellular densities (neurons plus astrocytes), for the signalling (Es) and nonsignaling (Ens) components. We found that fixed values of Ps and Pns were able to predict the entire range of behavioural states, from awake to sleep. The Ens term is constant over all states (at \( 2.94 \times 10^{16} \) ATP/(spikes per centimetre)) whereas the Es term depends on the neuronal firing rate driven by synaptic activity. The equation then provides the total energy expended in a region of the brain as a function of synaptic activity there. It is this equation that allows comparison between the absolute measure of energy utilization in a brain region and that calculated using the equation from the impulse firing, given by the last two rows in the table of Figure 14.

There are now (2020) over 100,000 papers using the technique of functional magnetic resonance imaging (fMRI), in which MRI is used in a configuration which allows blood flow to be indirectly measured non-invasively in an area of the brain of interest, called blood-oxygen-level
dependent (BOLD) contrast. As synaptically driven neuronal activity requires energy supplied by the blood in the form of glucose, the flow increasing or decreasing with the changes in energy demand, the BOLD measurement is proportional to the synaptic activity. One of the difficulties with the technique is, however, that the BOLD measurement does not give absolute measures, as there is no absolute baseline $S_0$, so that only proportional changes are available, designated $\Delta S/S_0$. This has led to the difficulty that as the amplitude of the BOLD signal $\Delta S$ is dependent on the un-measureable baseline $S_0$, then it might not be possible to compare changes in BOLD $\Delta S$ in different regions of the brain as each region might have a different baseline due to different on-going synaptic activity there. My wonderful senior colleagues Bill Gibson and Les Farnell, with whom I have worked with in mathematical modelling for over 30 years, showed a way forward was to combine the above equation with those for the BOLD signal, so providing an absolute measure of the dependence of the BOLD signal. Figure 15 shows how the BOLD signal due to a sudden increase (A) or decrease (B) in impulse firing ($\Delta<u>$) depends on the existing background firing ($<u_0>$), there being very large decreases in the signal $\Delta S/S_0$ for a given ($\Delta<u>$) as the background firing ($<u_0>$) increases. This work shows that decreases in the BOLD signal indicate a decrease in firing, with the negative BOLD signal becoming larger for a given drop in firing as the background firing decreases (Figure 15B).

It has been something of a mystery as to why BOLD signals take on such weird shapes as those shown in Figure 16 (Bennett et al., 2019). Our work indicates that this occurs because these signals arise as a consequence of mixtures of increased firing driven by excitatory synapses and decreased firing due to the action of inhibitory synapses, as Figure 16 shows. Of particular interest is the use of the above calculations to determine the extent of excitatory and inhibitory synaptic activity, measured using the BOLD technique, in different parts of the brain of patients suffering from schizophrenia, compared with normal subjects. Figure 17 shows that the difference can be explained in terms of a failure of inhibitory synapses in schizophrenia, pinpointing this as a major site for further investigation, as have other investigators using entirely different techniques, mostly applied to the brains of deceased patients.
Synaptic activity is the main energy requirement, with glucose metabolism measured in the brain using Positron Emission Tomography (from Table 3 in Hyder, Rothman and Bennett, 2013).

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<th>VGA</th>
<th>PRO</th>
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<td>0.69</td>
<td>0.84</td>
<td>0.99</td>
</tr>
<tr>
<td>calcCMR_{glc(ox)} (\mu mol/g per minute)</td>
<td>0.10</td>
<td>0.15</td>
<td>0.16</td>
<td>0.16</td>
<td>0.20</td>
<td>0.24</td>
<td>0.28</td>
</tr>
<tr>
<td>measCMR_{glc(ox)} (\mu mol/g per minute)</td>
<td>0.08</td>
<td>0.14</td>
<td>0.15</td>
<td>0.15</td>
<td>0.18</td>
<td>0.24</td>
<td>0.31</td>
</tr>
</tbody>
</table>

Details in Calculations. To convert the EEG data in Table 3 into units of $\Sigma (\delta, \eta, \xi)_{\text{spike}}$, simple conversions were needed with Eq. 6. The calculated CMR_{glc(ox)} [calcCMR_{glc(ox)}] was determined from Eq. 3. The measured CMR_{glc(ox)} [measCMR_{glc(ox)}] was determined from Eq. 4 assuming OGl of 5.6. AWK, awake; HAL, halothane; PRO, propofol; SEV, sevoflurane; SLP, non-REM sleep; VGP, acute vegetative; VGA, persistent vegetative.

*Details in SI Text, section 1 and see Fig. 1A for comparison between calcCMR_{glc(ox)} and measCMR_{glc(ox)}.
Figure 15. Fractional change in $\Delta S/S_0$ in BOLD signal as a function of the background firing rate and incremental firing rate, calculated with the Davis/HRB model. A: results for positive incremental firing above the baseline. In addition, the solid line shows the results according to the steady-state Friston model using equations 16, 22 and 21 with $v$ given by equations 5 and $E$ by equation 8 in the paper referenced below. Parameter values are $V_0 \, 0.02$, $E_0 \, 0.4, 0.32, 0.41 \, S^{-1}$. The neural activity $z$ in the Friston model is related to the incremental firing rate in the Davis/HRB model by $z5$. B: results for negative [ incremental firing rates below the baseline value] (from Bennett, Farnell and Gibson, 2018).
**Figure 16.** Plots of the blood oxygen level-dependent (BOLD) signal, S/S₀, as a function of time (t) for the various neuronal inputs, shown as a dashed line in each graph (scale on right axis). These have been calculated for comparison with the observations of Gonzalez-Castillo et al., (2012). The baseline firing rate is 1.2 and the Hyder-Rothman-Bennett (HRB)-Friston theory is used to calculate ΔS/S₀. A, B, C, and D correspond to graphs C104, C113, C102 and C109 respectively, in Fig. 4 of Gonzalez-Castillo et al., (2012). The color of each BOLD signal trace is the same as in that figure; the shaded blue area represents the duration of the visual stimulation task. (from Bennett, Farnell and Gibson, 2019).
Figure 17. Plots of the blood oxygen level-dependent BOLD signal, $\Delta S/S_0$, as a function of time ($t$) for various neuronal inputs $\Delta <u>$, calculated for comparison with the observations of Hanlon et al., (2016) on patients with schizophrenia (SP) compared with healthy control subjects (HC). The baseline firing rate is ($<u>$)$_0 = 1.2$, and the Hyder-Rothman-Bennett (HRB)-Friston theory is used to calculate $S/S_0$. In each case the neuronal input $\Delta <u>$ is shown as a dashed line of the same color as the corresponding BOLD signal (scale on the right axis): blue for healthy control subjects and red for schizophrenic patients. They compared with curves in Figure 2B of Hanlon et al., (2016): aDMN, A2 and PCUN. Note that excitatory impulse firing is not turned off, after -6s, to nearly the same extent in the schizophrenia patients compared with control subjects. aDMN, anterior node of the default mode network; A2, secondary auditory cortex; PCUN, precuneus. (from Bennett, Farnell and Gibson, 2019).
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brief action potentials and three electrical regions predicted to differentially control transmitter release. The Journal of Neurosci. 40(18), 3502-3516.


Summary of Discoveries on Neuroplasticity

Contents

1. The formation of synapses in embryonic development and following nerve injury
2. The plasticity of synaptic terminals for the release of transmitter substances
3. The retraction of nerves from their synaptic targets during development of the brain, the death of their cell bodies and neuron growth factors
4. The retraction of nerves from their synaptic targets on dendrites following stress, the collapse of dendrites and the loss of grey matter
5. The loss of functioning inhibitory synapses on neurons in the brain in schizophrenia

The question I asked over 50 years ago, is whether the neural connections made at synapses following embryonic development are fixed, or is the nervous system plastic, with synapses and the networks they subserve, coming and going throughout life. Some phenomenological experiments had been carried out on the nervous system that suggested this was the case but nothing was known at the level of the synapse.

1. The most accessible synapse for tackling the possibility of neuroplasticity is that between nerves and the muscles of the diaphragm and limbs, rather than those in the brain. In the 1970’s at Sydney I showed, together with a PhD student Alan Pettigrew, how the ends of nerves, which form a bulbous growth cone, on first reaching their target cells construct a terminal on them. The site at which this occurs becomes more specialized over time, with other growth cones approaching the muscle restricted to the original site. Two very interesting phenomena then occur. First, this formation of terminals at the site continues over a short period of days, to be followed by a period of regression of terminals over weeks until only one terminal remains at the original site. This was the first
description of neuroplasticity at the synapse and is now known to be related to maturing of neural circuitry, in which terminals win out for the synaptic site, contingent on their relative activity. The other phenomenon of interest is if nerves are injured once maturity has been reached, and regrow into the muscle from the site of injury, their growth cones only form synapses at the original site formed during early development. This indicates the existence of ‘recognition molecules’ at this site. The identity of such molecules has been a major task of molecular neurobiology since these observations were first made nearly 50 years ago, for such molecules hold out the promise of reconstituting injured or degenerating nerve connections.

2. But none of the above experiments elucidated if a single mature synapse was a dynamic structure, with ongoing changes that could even be observed at the structural level, rather than at molecular resolution. Two research students, Greg McLeod and Nick Lavidis, joined me in the work of examining if the relative ability of single sites of transmitter release was stable over time and we found they were not. More spectacularly however, was the discovery that new synapses could be forming and others regressing over periods of hours. This indicated very substantial synaptic plasticity in the mature nervous system.

3. Not only are nerve terminals to muscle eliminated during early development, but so are their neuron cell bodies in the spinal cord. The next question we tackled is whether there was nerve loss and that of their associated cell bodies in the normally developing brain. Now the eye and its retina are out-pockets of the brain during development, so the neurons in the retina of the eye, together with their individual axons that connect them via synapses to other neurons in the brain, are ideal for examining this question. I suggested to the visual system experts Bogdan Dreher and Ann Sefton that we could use relatively new techniques for uniquely labelling the neurons in the retina and their axons synapsing in the brain, and detect if the numbers varied. We found indeed that both axons and their neurons degenerated at a fast rate around birth, such that over 30% were lost. These observations indicated that
there is a quantitative loss of axon forming synapses and their neurons in the normally developing brain, even after birth, almost certainly associated with establishing the mature nervous system.

When during normal development neurons in the peripheral nervous system connect with muscle, neuronal survival is conditional on their obtaining a neuronal growth factor from the muscle. We argued that this should also hold for neurons in the brain, in our experimental model the retinal neurons: those that degenerate are those that fail to obtain a particular growth factor from, in this case, the brain to which they normally form synaptic connections. In order to identify a growth factor for these neurons we had to develop a technique for identifying the neurons in the retina that connect to the brain, the retinal ganglion cells, in a culture plate after their dissociation from the eye, which we did. That then allowed a PhD student, Mark Schultz, and I to take various molecular fractions of the parts of the brain to which these neurons normally connect and determine which molecules kept the neurons alive in the culture plate. To our surprise we discovered that a chondroitin sulphate proteoglycan from the brain was such a growth factor. Other more powerful growth factors have also been discovered for these retinal ganglion cells.

4. The next task I set myself was to determine if there was a naturally occurring loss of synapses deep in the brain following changes in behaviour. To this end I designed an experiment in which animals were confined in a small space for about 25 min per day for several weeks, which leads them to show stressful behaviour. Using magnetic resonance imaging (MRI) of their brains during this period showed that they lost grey matter in certain regions, such as the hippocampus required for memory. Subsequent autopsy of their brains showed that in these affected regions there had been a very large loss of synapses with a concomitant decrease in length of the dendrites on which these synapses normally connect, all painstakingly measured with histological techniques by a research student in my laboratory, Kassem. Furthermore, there was an exact quantitative match between the volume loss of grey matter determined with MRI and the volume loss of dendrites and
synapses determined at autopsy with histological techniques. This one experiment showed for the first time that a behavioural change can lead to the regression of synapses in the brain, and that grey matter changes directly reflect changes in synapses and their dendrites.

5. The brain uses up about 20% of the entire body’s energy, which involves the reaction of glucose with oxygen to produce adenosine triphosphate (ATP; the same molecule that Campbell and I discovered was a transmitter substance released from nerves in my very first research, described in project 1 below). Although we had shown that ATP could act outside of neurons as a transmitter at synapses, its principal role is inside the neuron, where, as in other cells it acts to provide energy. The most important utilization of this energy is that required to pump ions like sodium that are involved in the action potential, across the membrane. It occurred to me that there should be an exact quantitative relation between the number of active synapses generating action potentials in a region of the brain and the extent of glycogen used there. As labelled glycogen can easily be measured in the brain using non-invasive imaging techniques such as positron emission tomography (PET), it should be possible with this approach to determine the number of active synapses in any region of the brain one was interested in. Collaborating with colleagues in Yale, who were experts in using PET, showed that the hypothesis was correct. Subsequent work with my wonderful mathematical colleagues of some thirty years standing, Bill Gibson and Les Farnell, showed that the mathematical relation between energy utilization and the numbers of active synapses could be used to provide quantitative explanations for some here-to-for inexplicable phenomena. Chief among these were the ‘weird’ shapes for ‘blood oxygen level-dependent’ (BOLD) signals found in the very popular functional magnetic resonance imaging (fMRI) signals. These shapes were shown to arise as a consequence of mixtures of excitatory and inhibitory synapses. Perhaps the most interesting realization was that these ‘weird’ BOLD signals in the brains of patients with schizophrenia arise from a specific loss of inhibitory synapses,
providing further evidence that this loss is a major contributing factor to this dreadful malady.

It seems then appropriate, even if serendipitous, to have begun a research career with the discovery of ATP as a major transmitter substance released outside the cell (see project 1 below), involving among many other things the amelioration of thrombosis. Then to finish the career some 60 years later with using the action of ATP within cells to emphasize, non-invasively, that there is a loss of inhibitory synapses in schizophrenia.
Research Papers on Neuroplasticity
(Complete set)
(grouped according to each of the five sections of research described above).

1. The formation of synapses in embryonic development and following nerve injury

Post-synaptic Membrane Triggers Synapse Formation

Papers


**Summary Reviews**


**Motoneurone growth cones form synapses that are eliminated during development**

**Papers**


Summary Review

2. The plasticity of synaptic terminals for the release of transmitter substances

Non-uniform Probability of Quantal Secretion at different sites at Motor-Nerve Synapses

Papers


3. The retraction of nerves from their synaptic targets during development of the brain, the death of their cell bodies and neuron growth factors

Retinal ganglion cell death discovered during normal development

Papers
cells in the developing retina of the rat. *Brain Res. 255 (Dev. Brain Res. 3): 481-486.*


**Retinal ganglion cell growth promoting factors are supplied by different cellular sources**

**Papers**


Summary Reviews


4. The retraction of nerves from their synaptic targets on dendrites following stress, the collapse of dendrites and the loss of grey matter

Papers

Summary Reviews

5. The loss of functioning inhibitory synapses on neurons in the brain in schizophrenia

Papers


Part II ATP transmission at synapses

1. The electrical signs of inhibitory transmission to smooth muscles, and the identity of a novel transmitter substance, ATP ........................73
2. The electrical signs of excitatory transmission to smooth muscles, and the release of the transmitter substance ATP onto receptor clusters ........................79
3. Transmission to smooth muscles forming an electrical syncytium utilizing the novel transmitter ATP ...... 87
4. Transmission between astrocytes forming an electrical syncytium utilizing the novel transmitter ATP ......93

References ........................................................................................................101
Preface

As I describe in a brief autobiographical sketch (Introduction to *The Search for Knowledge and Understanding*), I had from the age of 16 been fascinated by the question of how does ‘consciousness’ arise, and had then come to the conclusion that the path to pursue the answer to the question was through a combined study of philosophy and nervous system/brain research. Having first graduated in electrical engineering, as determined by the edicts of my father, I set about to first attend lectures and tutorials in philosophy while at the same time carrying out research on the nervous system. It turned out that at the University of Melbourne there were just two outstanding researchers on this subject, Mollie Holman and Geoffrey Burnstock, who had combined their talents in using different electrical recording techniques to unravel some aspects concerning how the muscles of the internal organs, such as the viscera and vasculature, are controlled by nerves referred to as autonomic nerves (‘autonomic’ because they do not, in most cases, mediate willed commands). I joined them, and after being given necessary training in their electrical recording techniques was allowed free reign in pursuing research projects of my own choosing.
1. The electrical signs of inhibitory transmission to smooth muscles, and the identity of a novel transmitter substance, ATP.

I had the great good fortune to meet a senior PhD student in the laboratory, subsequently Professor Graeme Campbell. He introduced me to a beautiful smooth muscle, complete with its own component of the autonomic nervous system diagrammed in Figure 1. This shows individual neurons collected together beneath the muscle and projecting their nerves into the tissue in which they release transmitter substances to control the relative contraction or relaxation of the muscle, through releasing excitatory or inhibitory transmitter substances. I then set out to record the electrical signs of the inhibition by electrically stimulating these nerves and recording from individual muscle cells (some 3/1000 of a milli-meter in diameter) the results of the action of the transmitter on the cells. Such a recording is shown in Figure 2(a), which indicates that there is an increase in potential in the cell for about a second following a single stimulus, an increase called an Inhibitory Junction Potential (IJP). But what is especially significant about this figure is indicated in part (b), which shows that there is no significant change in the IJP in the presence of substances thought at that time to block transmission to all the muscles of the internal organs, indicating that the IJP was not due to such substances. So what was the identity of this substance? I discussed this at length in the laboratory with Graeme Campbell and the conclusion reached was that the only way to identify the substance was to follow the classical experimental protocols that had been used to identify the then known transmitter substances such as acetylcholine and noradrenaline. Graeme Campbell carried out these experiments and in doing so identified the transmitter substance responsible for the IJP as Adenosine Triphosphate (ATP). The detailed drama of this story has been described in Bennett (2013a; see also 2013b).

Given the very long time course of the IJP, the question which immediately arose is what determines such a time course? The standard explanations for the time course of synaptic potentials was that they are determined by the electrical properties of the muscle membrane, but this would not suffice in this case for if it were so then the time course could not be much longer than 0.1 second. The explanation for the time course had to wait several decades after the observation illustrated in Figure 1, indeed until the identification of the receptor molecules on the muscle that the nervously released ATP activated, called P2Y2 receptors (Abbracchio and Burn stock, 1994). In experiments with a number of
younger colleagues, receptors were shown to occur in clusters, and when ATP-like substances were released onto them they become internalized to the cell on which they reside (Figure 3). The time course of internalization was then shown to fit the time – course of decline of the IJP (Lemon et al., 2003, 2004), providing an explanation for the relatively slow time course of this synaptic potential.

Figure 1 Simplified diagram of a section through the taenia coli smooth muscle and the underlying tissue. A, Auerbach’s plexus of neurons; b, smooth muscle cell bundles; C, circular smooth muscle layer; c, connective tissue; E, serosal epithelium; G, ganglion cell; J, junction of muscle bundles; n, nerve bundle. (From Figure 7 in Bennett and Rogers, 1967).
Figure 2. NANC (Non-Adrenergic, Non-Cholinergic) transmission. Individual inhibitory junction potentials (IJPs) in a taenia coli muscle, recorded with an intracellular electrode, before (a) and after (b) bathing the muscle with cholinergic (atropine) and noradrenergic (guanethidine) blocking drugs. No change was observed in the characteristics of the IJP due to single stimulations, indicating that the nerves were not releasing acetylcholine or noradrenaline. This was the first identification of NANC transmission (from Fig. 12 in chapter 2 of MSc thesis of Bennett (1965); also see Bennett et al., 1966).
**Figure 3.** P2Y2-GFP in A7r5 cells under different experimental conditions. (A) Confocal images of A7r5 cells showing the clustering of P2Y2-GFP in the membrane 24 h after transection (a), and then the gradual loss of many of the clusters after exposure of the cells to 10 mM UTP for 1 min (b), 2 min (c) and 5 min (d); higher magnification inserts in a and d are taken from the solid square regions and show dense clusters of P2Y2-GFP in a that are largely lost in d. Note that in addition to the loss of many of the clusters, there is a gradual rounding up of cells in the presence of the agonist. (B) Confocal images of P2Y2-GFP as in A, but this time in the presence of monensin (5 mM); note no loss of P2Y2-GFP fluorescence, but the cells still round up. (C) Confocal images of P2Y2-GFP as in A, but this time in the presence of suramin (10 mM); note again no loss of P2Y2-GFP as in A, but this time in the presence of suramin (10 mM); note again no loss of P2Y2-GFP fluorescence, and in this case the cells do not round up. Calibration bar is 10 mm. (from Lemon et al., 2004).
1. **The electrical signs of excitatory transmission to smooth muscles, and the release of the transmitter substance ATP onto receptor clusters**

Although a satisfactory explanation for inhibition of muscle (the IJP) had been provided, the process of excitation, of an excitatory junction potential (EJP) had not. The sites of excitatory transmission can be delineated at the micro-level, as a research student Keith Brain and I showed that the sites of transmitter release along autonomic nerves, called varicosities, can be made to fluoresce on stimulation if they have previously been filled with a dye that fluoresces in the presence of calcium ions, for calcium enters nerve terminals when they are stimulated, as shown in Figure 4. This work first showed that all the varicosities allowed for the influx of calcium ions on a nerve being stimulated with a single pulse, although to various degrees (Figure 4). Second, it showed that following the influx of calcium ions it took hundreds of milliseconds for the calcium to decline to its original level before stimulation (Figure 5). The first of these observations indicates that as transmitter release varies as the fourth power of the calcium influx the probability of transmitter release should vary greatly between varicosities. The second of these observations indicates that the residual calcium in the varicosity will ensure that a second impulse within a few hundred milliseconds after a first will release more transmitter than the first, a process called facilitation. Both of these predictions proved to be true.

Subsequent to the discovery that the IJP is due to the release of ATP onto P2Y$_2$ receptor clusters it was found that in many smooth muscles that generate an EJP on nerve stimulation there is also a release of ATP (Westfall et al., 2002), only onto different receptor clusters, called P$_{2X1}$ receptor clusters (Figure 6). Determining the location of these clusters with respect to single varicosities showed that in many cases the clusters were co-localized beneath single varicosities, or occurred independent of varicosities and in a few cases varicosities occurred independent of any P$_{2X1}$ receptor clusters (Figure 6). These surprising results suggested that varicosity-located P$_{2X1}$ clusters were labile, and that in some cases ATP could be released onto muscle without receptors, so failing to give rise to an EJP at all.
Figure 4. Calcium concentration in varicosities following a short tetanus. Images of Oregon-BAPTA intensity in three varicosities of a single nerve terminal at 10 different times prior to, during and after stimulation at 5 Hz for 5 impulses. The impulse commenced at zero time, with the number giving the time in seconds. Every second image collected is shown. The calcium concentration increases during the train and then declines, following the end of the train, towards its resting concentration. Calibration bar is 2 um. In the scale at the right, the upper part corresponds to the greatest measured fluorescence intensity and thus the highest calcium concentration. The scale is linear with respect to the fluorescent intensity (From Brain and Bennett, 1997).
Figure 5. Changes in the calcium concentration, $\Delta[Ca^{2+}]$, in contiguous varicosities and inter-varicose regions after an impulse. A, shows a composite image of the chain of varicosities from which line scans are taken. Each frame in the composite was captured 10 to 15 s before the relevant line scan in order to record the location of the region being sampled. The horizontal continuous lines indicate the line sampled from when recording from a varicosity. The dashed lines indicate the locations of line scans through inter-varicose regions. The scale bar represents 2um. Some refocusing was required between frames in order to place the varicosity of interest in the plane of focus. B, the average change in $\Delta[Ca^{2+}]$ measured with line scans through 7 contiguous varicosities (A) and the six intervening inter-varicose regions (o), from A. Shown is the period from 20 ms before the impulse to 100 ms after the impulse. The fitted curve is the sum of two exponentials. C shows the same data set as in B, over a long time period of the declining phase on log-log co-ordinates. (from Brain and Bennett, 1997).
Figure 6. Confocal images of the distribution of P$_{2x1}$ receptor clusters with respect to single varicosities labelled with AbSV2 on smooth muscle cells in a region of the detrusor smooth muscle. In this double-label experiment, AbSV2 immunoreactivity is shown on the left (A), with AbP$_{2x1}$ immunoreactivity on the right (B). A merge of the two images is presented in a stereo view (C, D). In (A), the arrowhead points to an SV2-labelled varicosity not co-localized with a P$_{2x1}$ receptor-cluster representing about 4.5% of such clusters. The arrowheads in (B) point to large P$_{2x1}$ receptor clusters not co-localized with SV2 and associated with varicosities; the arrow points to an example of a small P$_{2x1}$ receptor cluster and associated with varicosities. The arrowhead in (D) points to an example of a group of large P$_{2x1}$ receptor clusters co-localized with varicosities. Calibration: 19 um. Images (E), (F) and (G) show individual varicosities with SV2 staining and P$_{2x1}$ staining. (E) shows a varicosity that appears to be almost surrounded by P$_{2x1}$ labelling. (F) and (G) show receptors that are confined to an ellipse that almost, but not quite, represents the projection of the varicosity onto the XY plane. Scale bars: 0.5 um. (H) is a low magnification view demonstrating the overall labelling observed in a cryosection of rat bladder detrusor. Calibration: 50 um. The boxed area in (H) is magnified in (I) and (J), which demonstrate the co-localization of SV2 and P$_{2x2}$ labelling of large varicosities on a nerve. (I) shows SV2 immunoreactivity and (J) shows P$_{2x2}$ immunoreactivity. Scale 5 um. (from Hansen et al., 1998).
3 Transmission to smooth muscles forming an electrical syncytium utilizing the novel transmitter ATP

It had been shown by Bozler (1941) in the first half of the 20th century that an action potential initiated at one site in a smooth muscle could propagate to sites millimetres away, distances many time the length of a single smooth muscle cell, so giving rise to the idea that that the muscle cells must be electrically coupled to each other. I set out to confirm this, and serendipitously showed that not only could the action potential propagate over relatively large distances but it could do so independent of the influx of sodium ions (Figure 7), requiring only an influx of calcium ions to propagate. This was the first calcium driven action potential to be discovered in cells, although changing the chemical environment of some cells, for example by adding tetra-ethyl-ammonium ions, can induce calcium action potentials (Tsien and Barrett, 2000-2003).

If then smooth muscle is effectively an electrical syncytium, it would be anticipated that an EJP recorded in one cell would be due to both ATP released onto that cell as well as that onto adjacent cells, with the currents generated by these propagating through electrical couplings into the cell recorded from. This being the case, local depolarization of a cell due to injection of current during an EJP would not affect its amplitude, as classical theory would expect, because the injection of current would be confined locally whereas the EJP arises from the more global release of ATP. This I showed to be the case (Bennett, 1967b), which I formalized in a model (Bennett, 1972, 1973), that was later elaborated in more detail with my colleagues (Bennett, Gibson and Poznanski, 1993), indicating how synaptic transmission mediated by ATP occurs in a syncytium of cells like smooth and cardiac muscle (Figure 8).

Most of the experiments and discoveries described above were made when I was still in my mid-twenties, and they significantly enhanced understanding of the structure and function of synaptic connections between autonomic nerves and the muscle of the internal organs. This was recognized by my being invited at 29 years by the then premier physiological society in the world, to write a Physiological Society Monograph (No.30; 1972).
Figure 7. Action potentials recorded in solutions of different sodium activity. Activity of the sodium in A, 118 mM; in B, 86 mM; in C, 25 mM. Note the rate of rise and overshoot of the action potential remains approximately the same at each sodium activity. (from Bennett, 1967a).
**Figure 8.** Diagram of a discrete bidomain model for the three dimensional smooth muscle syncytium. The x- and z- axes are in the transverse plane of the muscle and the y-axis is along the longitudinal axis of the muscle. Each filled node represents a point within a single smooth muscle cell, coupled to six other cells; the coupling is represented by continuous lines; the inside of each cell being coupled to the inside of four other cells in the transverse plane and to two other cells in the transverse direction; each of these lines represents the resistance of the intracellular pathway between adjacent cells. Each open node represents a point just outside single smooth muscle cells, coupled to similar points outside six other cells in the same configuration as that for the inside points; these are represented by discontinuous lines, each of which represents the resistance of the interstitial pathway between adjacent cells. The resistance of the intracellular and interstitial pathways is not necessarily the same in the x-, y- and z- directions. Each of the intracellular nodes is coupled to an adjacent intracellular node by parallel resistance and capacitance of the cell membrane. The figure shows the general structure of the discrete model (compare with Figure 23 in Bennett, 1972). Note that only a few nodes and their connections have been drawn. Theoretically, the system is infinite in the x- and y- directions and semi-infinite in the z-direction, with the muscle tissue occupying the region $z<0$. In a practical calculation, it is sufficient to take a maximum of 30 nodes in each of the x- and y- directions and 15 in the z-direction. (from Bennett, Gibson and Poznanski, 1993).
4 Transmission between astrocytes forming an electrical syncytium utilizing the novel transmitter ATP

Astrocytes also possess electrical junction coupling between each other in the brain and spinal cord. Although they also generate calcium waves in response to a mechanical stimulus, it was of interest to know if this wave was regenerative and could propagate hundreds of microns or even millimetres as does the regenerative calcium wave I had discovered in smooth muscle. We discovered by culturing discrete lanes of astrocytes that the non-regenerative calcium wave could propagate up to at least 0.5 mm along the lanes without diminution in amplitude (Figure 9). The mechanism by which this propagation occurred involved the release of ATP from one astrocyte onto P2y receptors of an adjacent astrocyte. This lead to the release of calcium from internal stores in the adjacent astrocyte, so allowing the calcium signal to be enhanced for propagation (Figure 10). Thus calcium signalling in an astrocyte network involves both electrical coupling through gap junctions as well ATP mediated transmission between the cells, as shown in Figure 11.
**Figure 9** Propagation of a $\text{Ca}^{2+}$ wave occurs between astrocyte lanes if these are not separated by distances of $>\approx$ about 140 um. (A, B) Parallel lanes of astrocytes in which the lane widths are 35- and 90-um, respectively, separated by cell-free lanes of 65- and 140-um, respectively; (s) astrocytes that gave a $\text{Ca}^{2+}$ response after mechanical excitation of the astrocyte indicated by the arrow. (C, D) Lanes of astrocytes that have been interrupted along their length by gaps of different lengths (73 um in C and 170 um in D); (s) astrocytes that responded with a $\text{Ca}^{2+}$ transient when the astrocyte identified by the arrow was mechanically stimulated; in panel C, the $\text{Ca}^{2+}$ wave propagated across the gap but in panel D it failed to cross the gap. Panels E and F give two different examples of the time taken for a $\text{Ca}^{2+}$ wave to propagate across an astrocyte lane and then to parallel astrocyte lanes separated by cell-free lanes; continuous lines, propagation across a lane, and the broken lines for the delay period before a $\text{Ca}^{2+}$ response was first observed on the edge of an adjacent lane. Calibration bar, for panels A–D, is 45 um (Bennett et al., 2006).
Figure 10  (A) Simplified schematic diagram of the steps leading from metabotropic receptor activation to Ca\(^{2+}\) release from the calcium store (endoplasmic reticulum) into the cytosol and ATP release from internal stores into the extracellular space. Transmitter (ATP) binds to the receptor which then interacts with the G-protein (G), leading to the replacement of GDP with GTP and the subsequent dissociation of the G-protein into subunits. The subunit G\(_a\) GTP binds to a site on phospholipase C-b (PLC) and this activated unit initiates an interaction with membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)), leading to the hydrolysis of PIP\(_2\) and the production of IP\(_3\). This diffuses into the cytosol where it opens IP\(_3\)-sensitive channels in the ER, allowing the release of Ca\(^{2+}\) into the cytosol. The IP\(_3\) is also assumed to interact with ATP stores inside the astrocyte, leading to the release of ATP into the extracellular space. An alternative pathway, involving diacylglycerol (DAG) and protein kinase C (PKC) is also indicated, though it is not used in the present model. (B) A lane of model astrocytes, represented by cubes of side 25 um, separated by spaces of width 25 um. The cubes have their centers in the xy plane (z \(\frac{1}{4}\) 0) and are aligned parallel to the x-axis. (C) A cube of side 25 um, representing a single astrocyte, subdivided into 5 \(\times\) 5 \(\times\) 5 cubes of side 5 um, as indicated by the broken lines. The grid points used in the numerical integration scheme are placed at the center of each sub-cube, thus giving 27 interior points and 98 surface points. At all points, the processes implemented are IP\(_3\) diffusion and Ca\(^{2+}\) release from the ER; at the surface points, the additional processes implemented are receptor activation, the G-protein cascade leading to IP\(_3\) production, and the release of ATP. The exterior space is likewise divided into sub-cubes of side 5 um with grid points at the centers and these are used to implement diffusion of ATP in the extracellular space (Bennett et al., 2005).
Figure 11 (A) Lane of model astrocytes, represented by cubes of side 25 um, separated by spaces of width 25 um. The cubes have their centers in the x, y plane (z 1/4 0) and are aligned parallel to the x axis. ATP diffuses in the extracellular space and binds to receptors that are uniformly distributed on the surfaces of the cubes. The neurons are not shown; although there is a one-to-one correspondence with the astrocytes, their interaction is via glutamate and, as explained in the text, it is not necessary to explicitly include glutamate diffusion in the model. Thus their spatial position is not needed for the calculations. (B) Neuron-astrocyte model network in which astrocytic transmission is effected by ATP and neuronal transmission by glutamate, as follows: from neuron to neuron, glutamate (GluC) acting on AMPA receptors; from astrocyte to neuron, glutamate (GluA) acting on NMDA receptors; from neuron to astrocyte, glutamate (GluB) acting on metabotropic receptors; from astrocyte to astrocyte, ATP acting on P2Y receptors. (C) Neuron-astrocyte model network in which astrocytic transmission is principally effected by GJs, together with a small component due to released ATP. (The ATP production rate is reduced to one-tenth of that in the model of part A.) The remainder of the network is as in part A (Bennett et al., 2008).
References


Summary of Discoveries on ATP Transmission at Synapses

Contents

1. The electrical signs of inhibitory transmission to smooth muscles, and the identity of a novel transmitter substance, ATP.
2. The electrical signs of excitatory transmission to smooth muscles, and the release of the transmitter substance ATP onto receptor clusters.
3. Transmission to smooth muscles forming an electrical syncytium utilizing the novel transmitter ATP.
4. Transmission between astrocytes forming an electrical syncytium utilizing the novel transmitter ATP.

As I recount in my recent book, *The Search for Knowledge and Understanding*, and above, I had become very interested in philosophy while an undergraduate in electrical engineering at Melbourne University. I was particularly fascinated with the question of what is the relation between the mind and the brain and of the origins of the stream of consciousness (as for example, delineated in the novels of Virginia Woolf and James Joyce). However, I soon realized that with gifts in philosophy substantially less than someone like Wittgenstein I had to tackle these problems obliquely, as it were. I thought this would be best managed by going into nervous system research, brain research if you like, and looking at the philosophical questions on the side. This I have now done for well over sixty years, resulting in some 400 papers in nervous system research and 10 books on the philosophy and history of brain research as related to mind and brain (see [https://www.amazon.com/M.-R.-Bennett/e/B001JS32MU](https://www.amazon.com/M.-R.-Bennett/e/B001JS32MU)).

I identified Professors Mollie Holman and Geoffery Burnstock as the best neurophysiologists then working in Melbourne and they accepted me as a PhD research student, giving me full reign to choose what seemed to me to be a good project. Here I struck gold, for they were the first to use electrical techniques to study the way in which nerves work in the Autonomic Nervous System that controls the internal organs, techniques that were readily mastered by me as a recent graduate in electrical engineering. Furthermore, I met a
brilliant fellow PhD student in their laboratory, Graeme Campbell, who was a master at dissecting nerves and muscles on which I could carry out electrical recordings of how the nerves control muscle cells.

1. My final, and greatest stroke of luck, was to choose a rare muscle that could relax on nerve stimulation, providing me with the possibility of carrying out the first investigation of the action of inhibitory nerves that relax muscle as a consequence of releasing a chemical substance onto the muscle at the points of contact between the two, called the synapse. What I observed on stimulating these nerves was a great surprise, namely that the electrical signs of nerve control could not be blocked with any known antagonist drug, indicating the existence of novel substances being released from the nerve at the synapse to relax the muscle. What was the identity of this substance? Campbell and I discussed various ways of identifying it and came up with a then old-fashioned approach, that in Campbell’s hands paid off and revealed that the substance was very likely to be Adenosine Triphosphate (ATP). Subsequently the receptor molecules on the muscle that ATP binds to when released from the nerves were identified. The clinical ramifications of the discovery of ATP at synapses in the last 60 years have been extraordinary, including for example the isolation of the major inhibitor of thrombosis.

2. I was able with research students, especially Keith Brain, to show how the nerve terminal was specialized to release ATP onto clusters of these receptors, and generate unique action potentials due to the influx of calcium ions into the muscle, with these potentials then propagating throughout the muscle. At the time of first discovering this ‘calcium action potential’ it was thought that all action potentials were due to the inward flux of sodium ions, that is only ‘sodium action potentials’ existed for which Hodgkin and Huxley had just won the Nobel Prize, so my discovery of ‘calcium action potentials’ was a big surprise.

3. One of the most complex aspects of studying transmission of ATP, or any other transmitter in smooth muscle, versus for example that to voluntary striated muscles, is that smooth muscles are very small in diameter and are electrically coupled to each other. That means that if an electrical signal is generated in one smooth muscle cell it propagates into adjacent muscle cells through the electrical junctions. This is referred to as the muscle cells forming an electrical syncytium. I was
able to show how transmitter released onto one cell gave rise to potentials that radiated into adjacent cells. Using mathematical modelling techniques I showed how the whole muscle, consisting of thousands of cells, integrated the effects of transmitter release onto some of them to give the observed physiological response.

4. Smooth muscle cells are not the only cells that form an electrical syncytium, as the major cell type in the brain and spinal cord is not neurons but glial cells called astrocytes that also form a syncytium. Furthermore, these cells also generate calcium transients, not through the passage of calcium into the cell across the membrane, as do smooth muscle cells, but by the release of calcium from intracellular stores in the cells. Very interestingly, in both cases of smooth muscle and glial cells the calcium transient is generated by the action of ATP on receptors located on the cell surface. My colleagues, especially Bill Gibson and Les Farnell and I designed experiments to see how far the calcium transient could propagate in the astrocyte syncytium, as well as the mechanism of generation of the transients. We also determined the role of gap junction couplings and how the release of ATP from the astrocytes contributed to the propagation of the transients. It was very satisfying to bring the level of understanding of the workings of the astrocyte syncytium up to that of the smooth muscle syncytium that I had first worked on over 50 years earlier.

One would hardly say that this research on the autonomic synapses was providing obvious insights into how the mind is related to the brain, to consciousness, but they did give me time for further reading, discussion and thought on the problem. This then lead me to conclude that a phenomenon like memory must involve long-term changes at synapses, the making of new synaptic connections and perhaps the loss of others, which is now called the plasticity of synapses. I then sought out a laboratory that was interested in such a possibility as well as in the general philosophical questions that still fascinated me. To my surprise I did not have to go to Harvard or Cambridge in 1968, but just to Sydney University, where Professors Peter Bishop and Liam Burke were researching. On approaching them that year I was accepted as a lecturer in Physiology, where I have been for the last 52 eventual years. That
story is given in Part I of this personal saga, which has given me so much
pleasure as well as the company and stimulation of great colleagues.
Research Papers on ATP Transmission at Synapses (Complete set) (grouped according to each of the sections of research described above).

1. The electrical signs of inhibitory transmission to smooth muscles, and the identity of a novel transmitter substance, ATP.

Discovery of Non-Adrenergic, Non-Cholinergic Transmission

Papers


Summary Reviews


2. The electrical signs of excitatory transmission to smooth muscles, and the release of the transmitter substance ATP onto receptor clusters

Quantal secretion of ATP occurs at single sympathetic varicosities

Papers


Reviews


Quantal secretion of ATP at sympathetic nerve terminals

Papers


Summary Reviews


ATP receptor clusters on smooth muscle and their dynamics

Papers


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Summary Review

3 Transmission to smooth muscles forming an electrical syncytium utilizing the novel transmitter ATP

Identifying the Autonomic Neuromuscular Junction

Papers


Summary Reviews
*Monograph of the Physiological Society No. 30*, pp271. Cambridge University Press, UK.

4 Transmission between astrocytes forming an electrical syncytium utilizing the novel transmitter ATP


**Backpiece** The growth of motor nerve terminal processes with respect to Terminal Schwann Cells (TSC) processes. Shown are the changes in terminal processes \((a, b)\), as well as their associated TSC processes \((c, d)\) over a time interval of 19 min in \(A\) and 52 min in \(B\). In each case, \(e\) and \(f\) give the superimposed images. In \(A\), a small terminal process appears between \(a\) and \(b\), and this follows the left-hand TSC process that has remained stationary over this time, as shown by \(c\) and \(d\). In \(B\), a small terminal process appears between \(a\) and \(b\), and this follows the central TSC process that shows little change over the time between \(c\) and \(d\). In \(A\), OG-5N was injected into the nerve terminal and AF568 into the TSC. In \(B\), AF488 was injected into the nerve terminal and AF568 into the TSC. Time stamps represent the time since capturing the first images of the nerve and TSC in the before–after series. The first images of filled TSCs were captured 5 hr 16 min \((Ac)\) and 3 hr 43 min \((Bc)\), respectively, after toads were killed. . (from Macleod, Dickens and Bennett, 2001).