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Biocytin-labelling and its impact on late 20th century studies of cortical circuitry

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Abstract

In recognition of the impact that a powerful new anatomical tool, such as the Golgi method, can have, this essay highlights the enormous influence that biocytin-filling has had on modern neuroscience. This method has allowed neurones that have been recorded intracellularly, ‘whole-cell’ or juxta-cellularly, to be identified anatomically, forming a vital link between functional and structural studies. It has been applied throughout the nervous system and has become a fundamental component of our technical armoury. A comprehensive survey of the applications to which the biocytin-filling approach has been put, would fill a large volume. This essay therefore focuses on one area, neocortical microcircuitry and the ways in which combining physiology and anatomy have revealed rules that help us to explain its previously indecipherable variability and complexity.

Keywords

Biocytin; Interneurone; Cortex; Circuitry; Hypothalamo-neurohypophysial system; Dual recordings

Introduction

Contemplating the contribution that Golgi made to neuroscience, there can be no doubt that in addition to his many outstanding, original findings and observations, he provided his contemporaries, most notably Cajal (Jones, 2007, for review) and many who came after, with one of the most important and influential neuroanatomical tools devised (Golgi, 1873). A huge body of work produced, with the Golgi method, from the last decades of the 19th century and on through the 20th century, documented a vast array of neuronal subtypes and, from many outstanding neuroanatomists of the day, some compelling insights into the development and organisation of the nervous system (for reviews, eg. DeFelipe, 2002; Fairén, 2007; Peters, 2007; White, 2007).

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What the Golgi method could not provide, however, was a direct link between neuroanatomy and neurophysiology, though the ideas and hypotheses, relating to function, that originated from purely anatomical studies, have at times been truly impressive. With the advent of intracellular recordings, in the middle of the 20th century, physiologists began to study the electrophysiological properties of excitable cells. Once it became possible to study central neurones under controlled conditions, *in vitro*, it rapidly became apparent that they did not all resemble the squid giant axon, nor did they all display the same properties (Llinás, Sugimori, 1980); Llinás, Jahnsen, 1982; Llinás, Mühlethaler, 1988; Llinás, 1988, for review).

In some brain regions, where a single neuronal class was predominant, it was possible to surmise the identity of the recorded neurones from their location. In other regions, multiple cell types existed, but physiologists fell into the unfortunate habit of classifying neurones according to a few very broad descriptions of firing properties. In cortical regions, for example, it became commonplace to assume that all the cells that displayed the unfortunately termed, 'regular spiking' behaviour were pyramidal cells, though not all pyramidal cells displayed this firing pattern (Connors *et al.*, 1982); similarly that all interneurones displayed so call 'fast spiking' behaviour. Despite the weight of neuroanatomical evidence for diverse sub-types of pyramidal cells and interneurones, we physiologists (*mea culpa*) tended to lump them together in our thinking, into just these two categories. Not surprisingly, these categories displayed huge diversity in their physiological properties, but without the appropriate tool(s) we could refine our thinking no further.

During the 1970's a number of intrepid labs developed methods for labelling intracellularly recorded neurones. One involved the intracellular injection of horseradish peroxidase (HRP) followed by histochemical processing to generate a permanent, dark, 'Golgi-like' reaction product (Kitai *et al.*, 1976; Cullheim *et al.*, 1976; Snow *et al.*, 1976). In the other, a fluorescent compound, lucifer yellow, was injected (Stewart, 1981). Although some beautiful work resulted from these methods, obtaining high quality recordings with sharp electrodes was difficult. HRP, being a large protein, rapidly blocked high resistance intracellular electrodes, while lucifer yellow, since it was most soluble as a lithium salt, dramatically altered the electrophysiological characteristics of recorded neurones (see also Tasker *et al.*, 1991). Neither gained widespread popularity with physiologists and many of us continued to record with only an approximate idea of what we were recording from. This limitation was particularly acute with dual intracellular recordings of synaptically connected cells when it became apparent that not all classes of synapse behaved in the same way (eg. Thomson, West, 1993; Thomson *et al.*, 1993a,b); synapse class being defined by both neurones, pre- and post-synaptic. One lab valiantly pioneered dual recordings in the hippocampus (Schwartzkroin, Mathers, 1978; Knowles, Schwartzkroin, 1981, Schwartzkroin, Kunkel, 1985; Scharfman *et al.*, 1990) with intracellular HRP or lucifer yellow labelling (Lacaille *et al.*, 1987), but the technical difficulties prevented many from following suit until Armstrong and colleagues developed the biocytin technique.

We take the opportunity that this retrospective essay gives us to tell the story of how Armstrong and Horikawa discovered and developed what has become a fundamental tool right across the neuroscience spectrum. In a nutshell, Armstrong's method became the late 20th century equivalent of the Golgi method.

Origins of the intracellular biocytin-labelling technique

William Armstrong's interest in intracellular recording and labeling stemmed initially from extracellular recordings of supraoptic or paraventricular nucleus neurones, which displayed a phasic bursting pattern of activity in hypothalamic slices (Hatton *et al.*, 1978), or acutely

prepared hypothalamic explants; a pattern that was very similar to that reported *in vivo* from vasopressin neurones (Dreifuss *et al.*, 1976; Brimble, Dyball, 1977; Armstrong, Sladek, 1982; 1985). In 1984, Armstrong joined the rapidly growing Anatomy and Neurobiology department at the University of Tennessee Health Science Center, to which many outstanding neurophysiologists had been recruited by Steve Kitai. Of particular relevance here, Kitai's lab was one of three that had independently, and near simultaneously, developed the HRP method of intracellular labeling (Kitai *et al.*, 1976; Cullheim *et al.*, 1976; Snow *et al.*, 1976). Encouraged by Hitoshi Kita, Mel Park, and in particular, by Charlie Wilson, Armstrong began intracellular recordings to see the events underlying phasic bursting and to differentiate between the properties of oxytocin and vasopressin neurons.

It rapidly became obvious that the cells needed to be labelled, firstly, because neurosecretory neurones had been historically recalcitrant to most Golgi stains, the promise of seeing a full dendritic arbor, or a possible axon collateral, in these neurons was enticing and secondly to distinguish between oxytocin and vasopressin neurones. Having a background in neuroanatomy and immunohistochemistry, Armstrong became attracted to the idea that intracellular biotin would be the best solution. After all, biotinylated secondary antibodies were the basis of the powerful avidin-biotin-peroxidase complex (ABC) approach and could also be used to couple any avidin-conjugated fluorophore. Accordingly, a number of biotinylating reagents were tried. Most were, however, poorly water-soluble and no labelling resulted from the amounts that could be dissolved in pipette solutions. Around this time Kyoji Horikawa, a neurosurgeon from Okinawa, joined Armstrong's lab and together they tried biocytin, a small and soluble complex of biotin and lysine.

The first attempt was successful. With 4% biocytin in the pipette, Armstrong and Horikawa recovered a supraoptic neurone after fixing the tissue with buffered 4% paraformaldehyde, further sectioning and labeling with avidin-fluorescein. Importantly, biocytin did not greatly alter the DC resistance or current-carrying capacity of the intracellular electrodes, and from then on, filling neurons became routine. Images similar to those from HRP-fills were generated by substituting the ABC method for fluorescence (Horikawa, Armstrong, 1988; Smith, Armstrong, 1990) (Figure 1.). A variant of the method using NeurobiotinJ ((N-(2-aminoethyl) biotinamide hydrochloride)), a chloride salt of biotin that can be iontophoresed more selectively with positive current at neutral pH, was published a year later (Kita, Armstrong, 1991).

In the early days, plastic embedded tissue was used for immunocytochemical double labelling. Serial 1Fm sections were cut through fluorescently labeled biocytin-filled neurones and immunohistochemistry for oxytocin- or vasopressin-associated neurophysins performed on adjacent sections to identify the neurone (Smith and Armstrong, 1993; Armstrong *et al.*, 1994). This was successful though tedious, and the more standard immunofluorescent double labeling methods in 50Fm sections, were reinstated following the success of Erickson *et al.* (1990) and Kawaguchi (1992). The characterization of differences in the electrical properties of oxytocin and vasopressin neurones (Stern and Armstrong, 1995; 1997) as well as the plasticity of both electrical and morphological properties in oxytocin neurons during pregnancy and lactation (Stern, Armstrong, 1996; 1998; Teruyama *et al.*, 2002) began in earnest.

The biocytin-labeling method was easily adapted to whole cell recordings from slices using lower pipette-concentrations of biocytin (*e.g.*, Kawaguchi, 1992), and for the Armstrong lab, this latter method continues to allow a much more detailed characterization of many membrane, synaptic and calcium-handling properties in identified vasopressin and oxytocin neurons (Stern *et al.*, 1999; Shevchenko *et al.*, 2004; Roper *et al.*, 2003; 2004; Teruyama

and Armstrong, 2007) as well as the plasticity of oxytocin neurones during pregnancy and lactation (Stern *et al.*, 2000; Teruyama, Armstrong, 2005; 2007; 2008).

The successful propagation of biocytin-labeling was clearly aided by its rapid adoption by colleagues in the department of Anatomy and Neurobiology at the University of Tennessee (Kawaguchi *et al.*, 1989; Wilson *et al.*, 1990; Kang, Kitai, 1990). During this period biocytin-labeling was able to reveal long axonal projections and terminal arborizations not fully appreciated with HRP labeling in cortex (Cowan, Wilson, 1994) or striatum (Kawaguchi *et al.*, 1990), and some of the first correlations of electrical properties and morphology of human cortical neurones (Foehring *et al.*, 1991), and of Cajal-Retzius and neurogliaform cells in layer one of neocortex (Hestrin, Armstrong, 1996). Further scientific impact of the biocytin method in a wide variety of neurons and systems is evinced by the more than 700 citations since its publication in 1988 (Web of Knowledge). In this article, we focus on its contribution to the understanding of cortical microcircuitry and in particular, the correlation between cell type and function.

Dual intracellular recordings with biocytin labelling

Publication of the intracellular biocytin labelling method coincided with Jim Deuchars, a very talented young neuroanatomist, joining the Thomson lab and this method, combined with dual intracellular recordings, soon became the signature of the lab (Deuchars *et al.*, 1994, 1996; Deuchars, Thomson, 1995a,b; Thomson *et al.*, 1995; 1996a,b; Ali *et al.*, 1998). Inspiration was needed to pursue this somewhat labour-intensive approach; 12-16 hour dual recording experiments, followed by 2-3 days histological processing and 1-2 weeks for neuronal reconstruction at the microscope. The inspiration came from two directions; the beauty of the neurones and the facility with which they could be identified, according to their morphology and cytochemistry (eg. Kawaguchi, Kubota, 1993; 1996; 1997; Han *et al.*, 1993) and the strikingly different properties that the different types of neocortical and hippocampal neurones and the synaptic connections between them displayed. There was also, of course, the unique excitement associated with watching and listening to two neurones 'talking to each other' and being able, subsequently, to see to whom we had been listening.

A purely anatomical approach to circuitry could, with considerable effort and not inconsiderable ingenuity, have documented the specificity in local circuitry that has gradually been revealed with dual recordings over the last two decades. This is demonstrated, for example, by the meticulous ultrastructural studies of White (see White, 2007, for review), who documented the very different frequencies with which a range of long distance afferent inputs to the neocortex innervate different types of cortical neurones. Anatomists studying cortical interneurones had also been able to document the subcellular compartments of pyramidal cells that were innervated by specific subtypes of GABAergic interneurones, using Golgi impregnation and electron microscopy, eg. the chandelier, or axo-axonic cell that selectively innervates the axon initial segments of pyramidal cells (Somogyi *et al.*, 1982). There were, however, limitations to this approach. In many cases it was not possible to identify the postsynaptic neurone(s) beyond, for example, its possession, or lack of, dendritic spines. With biocytin-filling it became possible to document the physiological properties of neurones that were subsequently identified anatomically and immunocytochemically (eg. Kawaguchi, Kubota, 1993; 1996; 1997; Buhl *et al.*, 1994; 1996; Hughes *et al.*, 2000).

Different patterns of transmitter release at synapses made with different postsynaptic neurones

The importance of the biocytin-filling approach to the development of this field is undeniable. Without a practicable approach to the identification of synaptically connected neuronal pairs, all we would have had would have been functional diversity, with no framework within which to explain it.

The observation that the synapses made by pyramidal cell axons with one type of postsynaptic neurone displayed a very different pattern of transmitter release from that displayed by its synapses onto another type of neurone, was perhaps the most controversial of the early findings from dual intracellular recordings (Thomson, West, 1993; Thomson *et al*, 1993a,b). Perhaps subliminal loyalty to the frequently misquoted principle proposed by Dale (1934) prompted disbelief in the possibility that two terminals from the same axon could behave completely differently, dependent upon the type of neurone that was on the other side of the synapse. Indeed, at least one of the eminent scientists, who were later to publish extensively on ‘depressing synapses’, confidently denied the existence of short-term synaptic depression at the time. These misconceptions were largely due to anomalies introduced by the use of extracellular electrical stimulation. This crude approach to the study of synaptic transmission and circuitry dominated much of the field for several decades and sadly remains extant. Suffice it to say that, with some notably valuable exceptions, such as the activation of the neuromuscular junction, or antidromic identification of projection neurones, this approach has led to many serious errors of interpretation and the development of dogma it has taken some time to dispel. What is being stimulated and how frequently, cannot be assessed, indeed, during a train of electrical stimuli, the number of axons recruited by the stimulus changes from stimulus to stimulus (Storm, Lipowski, 1994). Over the years, however, the basic principle, that the postsynaptic neurone influences the transmitter release machinery that is used by the synaptic boutons innervating it, has become widely accepted, though how this is achieved remains to be determined.

A simplistic summary of these differences in ‘synaptic dynamics’ is that some synapses have a high release probability (p) at rest and ‘depress’, in the short term, when repetitively activated. Others have a low p and facilitate. A large proportion of the terminals that constitute a high p connection have released transmitter in response to the first action potential (AP) of a train and have become refractory before the second (Betz, 1970). A smaller population of terminals is therefore available for the next release (Thomson, West, 1993; Thomson *et al*, 1993b, see also Tsodyks and Markram, 1997; Reyes *et al*, 1998; Chance *et al.*, 1998). At high p , ‘depressing’ synapses therefore, the second EPSP (excitatory postsynaptic potential) is, on average, smaller than the first and the third smaller than the second, in a train. In contrast, the synapses constituting connections that display a low release probability, rarely release in response to just one AP, but facilitate and may augment and potentiate during repetitive stimulation (eg. Thomson *et al*, 1993a; Thomson *et al*, 1995; Ali *et al*, 1998). At low p , ‘facilitating’ synapses therefore, the second EPSP, in a train is larger than the first and the third may be larger than the second. Careful analysis of responses to many different presynaptic firing patterns demonstrates that there is a great deal more to it than this (Thomson, 2003; Thomson, Lamy, 2007 for reviews; Thomson, West 2003; Bannister, Thomson, 2007 Brémaud *et al*, 2008) and some extremely complex patterns of release, that correlate with the types of neurones on both sides of the synaptic cleft, have been revealed.

Biocytin labelling allowed not only the identification of the interneurones receiving low ‘ p ’ facilitating inputs, but, since ultrastructural confirmation was possible, also provided an estimate of ‘anatomical n ’, i.e. the number of synaptic boutons from the presynaptic axon

that innervated the postsynaptic interneurone. Since total failures of transmission were common at these connections, a more compelling estimate of the very low release probabilities at these terminals (<1 per 100 APs) could be obtained than with fluctuation analysis alone (Deuchars, Thomson, 1995b; Thomson *et al*, 1995). For example, one synaptic connection exhibited more than 30% total failures of transmission, but involved between 6 and 12 synapses (6 of the 12 apparent at the light microscopic level were confirmed at the ultrastructural level), i.e. a release probability for each synapse between 0.06 and 0.12. This demonstrated that there was a fundamental difference between these terminals and those innervating other targets, like pyramidal cells.

Time course of the synaptic potential

The time course of an EPSP, or IPSP, has not inconsiderable functional significance. Rapidly rising events depolarise the spike initiation zone before significant Na⁺ channel inactivation can occur and spike activation by suprathreshold events has great temporal precision. Slower events sum more effectively, at lower frequencies, but by reaching spike threshold more slowly, may result in an increased spike threshold and a more sluggish and variable postsynaptic response. Similarly, fast inhibitory events contribute to synchronisation, by delaying firing amongst a population of postsynaptic neurones with temporal precision (eg. Cobb *et al*, 1995; Tamás *et al*, 2000).

Interneurons with narrow spikes and fast spiking behaviour (eg. parvalbumin-containing multipolar cells), receive fast EPSPs and IPSPs, while interneurons with broader spikes and adapting or low threshold spiking behaviour, (eg. somatostatin-containing dendrite-targeting interneurons), receive more slowly rising and decaying synaptic inputs. This is, perhaps, to be expected, since the electrophysiological characteristics of these different types of interneurons will determine to a great extent, both the duration of their action potentials and the time course of the synaptic events they receive. Moreover, different classes of interneurons express different glutamate receptors, the fastest interneurons expressing AMPA receptors with extremely fast kinetics (Jonas *et al*, 1994; Angulo *et al*, 1997; Geiger *et al*, 1995) and fewer NMDA (N-methyl-D-aspartate) receptors (Níryi *et al*, 2003).

Somewhat more surprising, however, was the finding that the presynaptic interneurons' characteristics also correlate with the duration of the IPSPs that they elicit in postsynaptic pyramidal cells (Thomson *et al*, 1996c). Since some of the more slowly rising IPSPs are elicited by dendrite-targeting interneurons, part of the difference in IPSP time course (recorded at the soma) can be ascribed to dendritic filtering. However, CCK- (cholecystokinin-) containing basket cells deliver slower IPSPs than parvalbumin-basket cells, while hippocampal Ivy cells (Fuentesalba *et al*, 2008) deliver more slowly rising IPSPs than more distally targeting bistratified cells.

Thus, everything to do with fast spiking cells, such as parvalbumin-basket cells, is fast, their APs and their inputs and outputs. This includes axon conduction, since these axons are strongly myelinated and ideally suited, therefore to synchronise many other neurones over several hundred microns. Everything to do with slow interneurons, like bipolar and Ivy cells, on the other hand, is slow. Interestingly, as the properties of all cortical neurones become faster during development, the synaptic events also become faster, the entire system 'speeding up' in parallel (Ali *et al*, 2007).

Binomial parameters

By this time we knew that the dynamic properties of synaptic connections and the time course of the EPSPs and IPSPs elicited were strongly correlated with the types of neurones involved in the connection. To take these comparisons a step further and compare different

types of synaptic connection and the same connections under different circumstances, it was important to have a simple model that described basic parameters. The binomial model of transmitter release (Katz, 1996, for review) provides an adequate description of the statistics of transmitter release at many of the synapses made by pyramidal axons. In brief, with three parameters defined (n , the number of release sites; p , the probability that each release site will release in response to an action potential; q , the quantal amplitude), the probability that a synaptic event of a certain size will occur can be calculated, as can the mean amplitude of many such events ($M = n.p.q$). Many studies of these synapses had reported wide mean amplitude ranges, which might result from differences in any, or all of these parameters. However, when connections for which both the pre- and the post-synaptic neurone could be identified were analyzed, this wide diversity was found to represent several much more discrete distributions. For example, pyramid to pyramid connections in layer 3 displayed a larger q than those in layer 4 or layer 6, but a smaller q than those in layer 5. Within any one population, the most variable parameter was usually n . The parameters p (at low frequencies) and particularly q , typically fell within narrower limits. This rather begs the question of by just how much synaptic plasticity can alter p and q , both of which are reported to be significantly modified (to differing degrees) in various forms of lasting synaptic potentiation and depression. Either plasticity is very limited in the young adult rat and cat (from which these data were obtained), or the preparation of brain slices returns all synapses to a basal state, or the number of synapses involved in a connection, rather than their individual strengths, or release probabilities, is the main parameter affected in a lasting way.

Again, an additional level of specificity is seen in layer 6. All layer 6 pyramid to pyramid connections displayed a similar q , but the parameter p was much lower for pairs in which the presynaptic neurone was a cortico-thalamic pyramid, rather than a cortico-cortical pyramid (Brémaud *et al*, 2007). This correlates with the depressing EPSPs elicited in other pyramidal cells by cortico-cortical pyramids (Mercer *et al*, 2005) and the facilitating EPSPs elicited by cortico-thalamic pyramids, both in other pyramidal cells and all types of interneurons studied, including parvalbumin-containing cells (Thomson *et al*, 2006). To date, the layer 6 cortico-thalamic pyramid is the only neocortical pyramidal cell type shown to elicit facilitating EPSPs in all postsynaptic target cell types, even those that receive depressing inputs from other presynaptic neurones (see also Tarczy-Hornoch *et al*, 1999, for layer 4; Alexander, Godwin, 2005; Granseth, Ahlstrand, Lindstrom, 2002 and Castro-Alamancos, 2002, for cortico-thalamic connections; Thomson, 2010, for review).

Again, without the accurate identification of both pre- and post-synaptic neurones that is possible with paired recordings and biocytin-labelling, these distinct differences between components of the local circuitry would remain blurred and could not be attributable to identifiable cell classes.

Postsynaptic receptor subtype

With the addition of pharmacology, dual intracellular recordings with biocytin-labelling were also able to demonstrate a previously unsuspected postsynaptic receptor specificity that was dependent upon presynaptic identity.

Synaptic GABA_A receptors (GABA_ARs) are pentameric receptors, typically consisting of two α -subunits, two β -subunits and a γ 2-subunit. Hippocampal pyramidal cells express up to 10 subunits (Nusser *et al*, 1996): five α -subunits (α 1-5), three β -subunits (β 1-3), a γ 2- and a δ -subunit. The latter, together with α 4 (and β -subunits), forms extrasynaptic receptors, but there remain many possible pentameric combinations of the eight other subunits. Pharmacological studies demonstrated that the synapses innervated by one type of

presynaptic interneurone utilise one type of GABA_AR, while another type of GABA_AR is used by neighbouring synapses that are innervated by a different class of interneurone (Pawelzik, *et al* 1999; Thomson *et al*, 2000; Ali *et al*, 2008).

A particularly striking example is seen in the $\alpha 1\beta 2/3\gamma 2$ -GABA_ARs innervated by parvalbumin-containing basket cells and the $\alpha 2\beta 2/3\gamma 2$ -GABA_ARs innervated by CCK-containing basket cells, in hippocampus and neocortex. Although it was possible to confirm some of these findings with immuno-gold labelling at the ultrastructural level (Níyri *et al*, 2001), the original finding and the ‘follow-up’ in neocortex, relied on paired intracellular recordings with pharmacology and biocytin-labelling. In contrast, both in neocortex (Ali *et al*, 2008) and hippocampus (Thomson *et al*, 2000), specific types of dendrite-targeting interneurons (including neocortical Martinotti cells and hippocampal bistratified cells) innervate $\alpha 5$ -GABA_ARs. The potential importance of this specificity was demonstrated by behavioural studies in mutant mice. In each group of mice, one α -subunit was rendered insensitive to benzodiazepines (but otherwise entirely normal) and the effects of benzodiazepines on their behaviour tested. Later psychopharmacological studies confirmed some of these findings. In brief, $\alpha 1$ -GABA_ARs mediate the sedative effects of benzodiazepines, $\alpha 2$ - or $\alpha 3$ -GABA_ARs mediate their anxiolytic effects, while $\alpha 5$ -GABA_ARs appear to control the acquisition of some types of memory, since partial blockade of these receptors enhances aspects of cognitive performance in rodents and people (Möhler *et al*, 2002; Thomson, Jovanovic, 2010, for reviews). It is therefore possible to identify the interneuronal subtypes that may contribute to the control of different behaviours and mood states.

Selectivity in connections

At the time that paired recordings in neocortex began, there was little interest in the field as to whether there were any rules governing which cells were synaptically connected. Despite White’s meticulous studies (2007), clearly demonstrating the specificity of connections made by incoming axons, the dogma at the time was that local circuit axons made synaptic contacts equally readily with any cell that happened to be near enough. Large studies based on single cells filled beautifully *in vivo* and reconstructed (eg. Binzegger *et al*, 2004;Stepanyants *et al*, 2008) estimated connectivity rates for different potential targets based on the number of each type of cell in the region or layer, its dendritic length, or surface area in that layer and the trajectory of the potential presynaptic axon. The estimates were made with the assumption that there was no selectivity in the connections that an axon made (or that a dendrite accepted). A, perhaps not surprising prediction of such studies, if boutons were to be distributed indiscriminately across all available targets, is that the number of boutons involved in a connection between two neurones was very small, typically one.

That scientists wish occasionally to take short-cuts is perhaps understandable, especially when years of labourious paired recordings with neuronal reconstructions, or extensive anatomical studies are the alternatives available and when the community is thirsty for numbers to plug into their model or theory. Needless to say, however, predictions from White’s previous findings (White, 2007, for review) have proved rather more accurate. We are far from documenting all the specificity that is present in these cortical circuits, but, when paired recordings are performed to identify the synaptic connections and the recorded cells are identified anatomically, it rapidly becomes apparent that some types of connection are common, while others are rare enough to be considered non-existent. This is the case even when the axonal arbour of the potential presynaptic neurone overlaps the dendritic arbour of the potential postsynaptic neurone in space. For example, layer 4 spiny cells densely innervate layer 3 pyramidal cells (Feldmeyer *et al*, 2002), but layer 3 pyramids do

not reciprocate, despite the long apical dendrites of layer 4 pyramidal cells that ascend through the local axonal arbours of layer 3 pyramidal cells. Layer 3 pyramidal cell axons do not avoid all layer 4 cells, however, they may not innervate pyramidal, or spiny stellate cells in layer 4, but they do excite interneurons with cell bodies in layer 4 (Thomson *et al*, 2002b; Thomson, Bannister, 2003; Ali *et al*, 2007).

A similar specificity is seen between layers 3 and 5. Layer 3 pyramids densely innervate layer 5 pyramids, but layer 5 pyramids rarely, if ever innervate layer 3 pyramids, even though their axons can extend to the superficial layers. Moreover, the descending layer 3 axons are quite selective about their layer 5 pyramidal targets. They rarely innervate the smaller, shorter pyramidal cells, focussing their attention on the large tufted cells whose apical dendrites run close to their own (Thomson, Bannister, 1998). Another example of very local target selection is seen in layer 6. The cortico-cortical cells innervate all types of pyramidal cells in layer 5 and 6, apparently indiscriminately (though further work is needed). They rarely, however, innervate interneurons (Mercer *et al*, 2005). In striking contrast, layer 6 cortico-thalamic pyramidal cells rarely innervate other pyramids, preferentially innervating interneurons in the deep layers (West *et al*, 2006; Thomson, 2010, for review). What their preference is in layer 4, remains somewhat controversial.

A finer, sub-compartmental level of specificity

That interneurons innervate very specific regions of their postsynaptic targets has been known for a long time. That pyramidal cells might be equally selective has not excited the same degree of interest. A number of studies do, however, indicate that this may be the case (Spruston, 2008, for review). For example the axons of excitatory layer 4 cells selectively innervate the basal dendrites of layer 4 pyramidal cells and layer 3 pyramidal cells (Feldmeyer *et al*, 2002; Thomson *et al*, 2002; Bannister, Thomson, 2007). This is not because layer 4 spiny cell axons do not extend far enough to innervate the apical dendrites of their targets; in fact, there is a significant dorso-ventral region of overlap between the basal and oblique dendrites of layer 3 pyramidal cells. In contrast, layer 3 pyramidal axons also contact the apical oblique dendrites of other layer 3 pyramidal cells. In addition, inputs from layer 3 were found, on average, to be further from the soma than inputs from layer 4 ($97\mu\text{m}$ cf. $69\mu\text{m}$) (Feldmeyer *et al*, 2002) and to display different dynamic properties (Bannister, Thomson, 2007).

Evidence for specific sub-compartment targeting can also be obtained from analysis of the electrophysiological characteristics of synaptic connections. The image that emerges from a number of studies is that the inputs from one specific source, be it local or long distance, occupy a region of dendritic space that forms the wall of a hollow shell, at a certain electrotonic distance from the soma, upon which it is centred (in layer 5, Thomson *et al*, 1993; Markram *et al*, 1997; layer 4, Bannister, Thomson, 2007, layer 6, Mercer *et al*, 2005; layers 3-6, Brémaud *et al*, 2007, and in CA1, Deuchars, Thomson, 1996). In consequence, some inputs will always be further from the soma than others and be subject to different degrees of dendritic filtering, dendritic inhibition and voltage-gated channel modulation. They will, moreover, sum in a more spatio-temporally prescribed way with other types of inputs than random connectivity would allow. For example, some combinations of inputs will always come into different dendritic branches and reach the soma independently, summing there linearly. Others will arrive in the same branch, one type more distal than the other and the way in which they sum may be determined by which one arrives first (Branco, Häusser, 2009).

The biocytin-labelling technique links physiology and anatomy

One of the greatest advantages provided by biocytin-labelling has been the ability to link the information gained from a wide range of physiological and pharmacological studies with that gained from seminal anatomical studies. Long before cells were filled intracellularly, many different types of neurones had been described in detail, many similarities and differences across species and during development had been documented and changes in disease observed. Cell class identification allows data obtained from different types of studies to be correlated directly. For example, *in vivo* recordings (with juxta-cellular labelling) that demonstrate how different classes of interneurons behave during different hippocampal rhythms (Klausberger *et al*, 2003; 2004; 2005; Somogyi, Klausberger, 2005; Tukker *et al*, 2007), can be correlated with a large body of high quality anatomy and immunocytochemistry, local and long distance circuitry data and through psychopharmacology and cell type specific genetic manipulation studies, to behaviour.

Many, more recently developed approaches still rely on biocytin-Avidin-HRP for a permanent reaction product and for detailed reconstructions and ultrastructure of neurones recorded intracellularly. Mice can be genetically engineered to express green fluorescent protein (GFP) in specific subsets of neurones, so that, for example, the connections between one class of GFP-labelled interneurone and a pyramidal neighbour can be targeted experimentally. However, a permanent histological record of the pairs recorded still relies on intracellular labelling and processing. There are now ranges of fluorescent dyes that are great improvements on the days when lucifer yellow was the only option and many hope that improvements in optics and software will allow automated reconstructions of filled neurones. We are not quite there however. While essentially two-dimensional cultured neurones, with few structures in the way of the light-path, can be accurately reconstructed with confocal microscopy, few axonal arbours in thicker sections, that contain many other neurones, glia and myelin can be reliably reconstructed. Furthermore, automated reconstructions demand multiple Z-stacks that must be stitched together in 3 dimensions to reconstruct neurones with the same quantitative granularity achieved by a computer-based manual tracing system, a feat not routinely available in commercial software packages. It should also be remembered that manual reconstructions teach the operator a lot about the neurone(s) under the microscope, something that will be lost if/when reconstructions become entirely automated.

The future

It seems unlikely, in this translational age, that many large studies of the finer details of microcircuitry will be supported, despite the many important questions that remain to be addressed and the many examples of inadequate information leading to misconceptions that drive poor experimentation and woolly thinking. However, more recently developed approaches may help. Novel and technically sophisticated protocols often have more impact than more traditional approaches and may fuel a resurgent interest in functional circuitry.

In a recent study in mouse barrel cortex, Channelrhodopsin-2 (ChR2, a light-sensitive depolarising ion channel) was expressed in five different presynaptic cell populations, in different groups of mice. Action potential propagation was blocked pharmacologically, so that only those synaptic boutons at the centre of a focussed light beam could be activated by ChR2 to release glutamate. A single layer 3 pyramid was recorded whole-cell, light pulses were activated randomly over a grid centred on the postsynaptic soma and the grid positions at which light could evoke postsynaptic responses in that layer 3 pyramid were recorded. Thalamic afferents were found to contact ventral portions of basal dendrites. Inputs from layer 4 excitatory cells also contacted basal dendrites, but were more superficial than

thalamic inputs. Axons of other layer 2/3 pyramids innervated upper basal and apical oblique dendrites, while inputs from primary motor cortex targeted the dendritic tuft in layer 1 (Petreanu *et al*, 2009). This clever approach may largely have confirmed existing evidence, but could be further refined and extended to answer a number of outstanding questions about circuit specificity.

Another recently introduced technique may allow rapid strides in circuitry research (Callaway, 2008, for review). A modified rabies virus is injected into a single neurone *in vivo* and that cell (and only that cell) is provided with what the virus needs if it is to transfer to another neurone transynaptically. Many, if not all the cells that are presynaptic to that single infected cell become infected (infection success possibly being dependent upon region and cell type). The virus triggers production of GFP (and/or some other protein) in the presynaptic neurones, which can then be identified and/or manipulated. It is also feasible to limit the types of neurones so affected. This approach could document many more synaptic connections than dual intracellular recordings, in any given time, but would also rely on high quality anatomical analysis. The limitation will eventually be that only one postsynaptic cell is identifiable and that this one cell, even if the virus does not affect it too seriously, will support only a limited number of functional investigations. All the presynaptic cells could, in principle, express Ch2, for example, but only a very few could be tested for the functional properties of their inputs onto that one postsynaptic cell.

Meanwhile, advances in confocal and multi-photon technology and increasingly elegant ways of labelling molecules with fluorescent and electron-dense molecules, together with the advances in molecular biology and the many different types of genetically modified mice becoming available, offer other ways to combine functional with structural studies of the brain. In the previous Cajal Club special edition (Swanson *et al*, 2007), several authors discussed what might be seen as the demise of traditional neuroanatomy. In an attempt to end on a more optimistic note, we should remember that although traditional anatomy (and biocytin recovery probably now falls into that area) may not always be published in the highest profile journals, or receive the most citations in year one following publication, it lives for a very long time.

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Abbreviations

ABC	avidin-biotin-peroxidase complex
AMPA	α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
AP	action potential
CCK	cholecystokinin
Ch2	channelrhodopsin
EPSP	excitatory postsynaptic potential
GABA	γ -aminobutyric acid
GABA_AR	GABA receptor type-A
GFP	green fluorescent protein
HRP	horse radish peroxidase
IPSP	inhibitory postsynaptic potential
M	Mean amplitude of the E/IPSP
<i>n</i>	the number of release sites
<i>p</i>	the probability that each release site will release transmitter in response to an action potential
<i>q</i>	the quantal amplitude
NMDA	N-methyl-D-aspartate
VIP	vasoactive intestinal polypeptide

Figure 1.

A biocytin-labelled supraoptic neurone from the original Horikawa and Armstrong (1988) study, demonstrating the permanence of this method if the tissue is appropriately mounted.