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CLASSICS

Neurotransmission: peptide transmitters turn 36

Michael Nusbaum discusses the impact of Lily Yeh Jan and Yuh Nung Jan's classic paper 'Peptidergic transmission in sympathetic ganglia of the frog', published in The Journal of Physiology in 1982.

The ability of neurons to communicate via the regulated release of neurotransmitter was first established almost a century ago (reviewed in Loewi, 1945). The first few decades of neurotransmitter identification were focused on the 'small molecule transmitters', such as acetylcholine (ACh), noradrenaline (norepinephrine), serotonin, dopamine, GABA and glycine. Given this long-established pedigree for neurotransmitters, it might be surprising to some readers that it was only 36 years ago that Lily Yeh Jan and Yuh Nung Jan first determined that a peptide could also (a) be a neurotransmitter, (b) affect target neurons at a distance from their release site (i.e. no close apposition synapse required) and (c) very likely be released as a co-transmitter with a small molecule transmitter (in this case, ACh) (Jan and Jan, 1982). Fast forward to 2017 and peptide transmitters are by far the single largest group of transmitter molecules, containing well over 100 members (van den Pol, 2012). In comparison, there are still fewer than a dozen classical small molecule transmitters. Even the small stomatogastric ganglion (26 neurons plus ~20 inputs) of the crab, *Cancer borealis*, contains at least 60 peptide (co)transmitters, many of which are known to modulate the motor circuits therein (Marder, 2012; Chen et al., 2014; Nusbaum et al., 2017). The seminal finding of transmission 'at a distance' has also stood the test of time and is now a general principle of peptidergic transmission, as is the co-localization of peptide and small molecule transmitters (van den Pol, 2012; Nusbaum et al., 2017).

To set the scene: by 1980, the concept of peptides as neurotransmitters, their co-localization with small molecule transmitters and the associated concept of neuromodulation were already in the literature (Kupfermann, 1979; Snyder and Innis, 1979; Hökfelt et al., 1980). However, prior to Jan and Jan's 1982 paper, these concepts were not yet fully established at the level of identified neurons and synapses. The fact that co-transmission, in particular, was already evident nearly four decades ago likely is surprising to some because, despite a smattering of subsequent studies (Adams and O'Shea, 1983; Sigvardt et al., 1986; Bishop et al., 1987; Whim and Lloyd, 1989; Kupfermann, 1991; Nusbaum et al., 2001), it has only been during the past 10 years or so that the functional consequences of co-transmission have become a growth industry (Vaaga et al., 2014; Nusbaum et al., 2017).

The lag between the earlier discovery of putative peptide transmitters and their subsequent documentation as neurotransmitters was not due to a dearth of data about their presence and anatomical localization, as immunocytochemistry was already a well-established tool for this purpose (Snyder and Innis, 1979; Hökfelt et al., 1980). This lag, instead, resulted primarily from technical limitations which prevented single neuron-level electrophysiological access to the putative peptidergic neurons and their identified postsynaptic targets. This approach was especially challenging in the mammalian central nervous system (CNS) which, at that time, was not the friendly territory for synaptic physiologists that it has more recently become. The study of peptidergic transmission involving identified neurons is also vexing because the postsynaptic targets are not obvious, precisely because this type of transmission may lack the point-to-point synaptic contacts and precise postsynaptic potentials that are the hallmarks of 'classical' ionotropic synaptic transmission mediated by small molecule transmitters (Nässel, 2009; Nusbaum et al., 2017).

By the late 1970s, Stephen (Steve) W. Kuffler, a senior investigator at Harvard Medical School, had an exceptional track record for identifying and using different experimental systems to address different unresolved issues in neurobiology, and the issue of peptidergic neurotransmission was on his to-do list (Kuffler, 1980; McMahan, 1990). Kuffler's approach was sensible and simple; namely, go somewhere more accessible than the mammalian CNS, such as the bullfrog *Rana catesbiana* autonomic nervous system (Kuffler, 1980). The selection of sympathetic ganglia as the experimental system provided the advantages that (a) the somata are relatively large (important for obtaining good-quality intracellular recordings), (b) it was easy to distinguish between the two principal classes of neurons (B and C neurons) based on soma diameter and (c) the B and C neurons lack dendrites; they receive synapses directly onto their soma and the proximal axon segment.

Jan and Jan [now highly regarded senior scientists at the University of California, San Francisco (UCSF)] were postdocs with Kuffler when they elected to begin work on this project in May, 1978. In a remarkably short period, they published a compelling series of brief papers elucidating various aspects of peptidergic transmission in the frog sympathetic ganglia (Jan et al., 1979, 1980a,b), which was briefly outlined in a personal account of how the project developed in a compilation of endearing remembrances of Kuffler by his colleagues (McMahan, 1990). After moving to their new faculty positions at UCSF, they continued this work and soon thereafter published the foundational paper that firmly established peptides as neurotransmitters (Jan and Jan, 1982). Specifically, using an impressive array of techniques whose results are displayed in 17 figures (there were no 'supplemental figures' in those days), they showed that a mammalian LHRH (luteinizing hormone-releasing

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hormone)-like peptide was a neurotransmitter released by spinal neurons presynaptic to one of the two types of principal cells (C neurons) in the two caudal-most ganglia of the bullfrog sympathetic chain (see fig. 15 in Jan and Jan, 1982).

Despite the lack of LHRH-like peptide-containing synapses onto the other principal cell type (B neurons), both B and C neurons exhibited the same peptide-mediated depolarizing response following stimulation of the neurons presynaptic to C neurons. Equally noteworthy was that this stimulation also elicited ACh-mediated excitatory postsynaptic potentials (EPSPs) only in the C neurons. These results, summarized in fig. 15 of their 1982 paper, collectively supported the hypothesis that the LHRH-like peptide was released proximal to C neurons and then diffused for a distance considerably further than that encompassing the classical synaptic cleft to bind to peptide receptors present on the B neurons.

At the time the Jans were working on this project, there was a long-standing list of criteria that were expected to be fulfilled for a molecule to be formally designated as a neurotransmitter in a particular neuron (Jan and Jan, 1982). These criteria included validating that a transmitter candidate was (a) present in the neuron of interest (particularly in its presynaptic terminals), (b) synthesized therein (but see Tritsch et al., 2014), (c) released in an extracellular Ca^{2+} entry-dependent manner (but see Shakiryanova, 2011), (d) removed from the postsynaptic site by transporter-mediated uptake, diffusion or enzyme-mediated degradation, and that its physiological actions (e) mimicked in detail those of the native transmitter and (f) were suppressed by a receptor antagonist of the native transmitter. Just prior to the work by the Jans and Kuffler, many, but not all, of these criteria had been met for the peptide substance P in the spinal cord and hypothalamus (Iversen et al., 1976; Otsuka and Konishi, 1976).

Documenting these criteria was pivotal to this foundational effort verifying that a LHRH-like peptide was indeed a neurotransmitter (Jan and Jan, 1982). This was accomplished by combining immunolabeling, radioimmunoassays, nerve transection/transmitter depletion experiments and electrophysiology. As part of this effort, the Jans showed that directly applied mammalian LHRH and several synthetic analogs, but not substance P (which also depolarized the same sympathetic neurons and was immunolocalized to the same ganglion), mimicked the actions of the peptide transmitter.

Several additional events that are now standard occurrences at most, if not all, peptidergic synapses were also documented in this study (Jan and Jan, 1982). These events, now collectively bundled as common characteristics of modulatory/metabotropic actions, are distinct from those at ionotropic synapses mediated by small molecule transmitters (side note: the latter can also have modulatory actions, by binding to different receptors, and there are a few examples of peptides having ionotropic actions). Specifically, Jan and Jan found that (a) single presynaptic action potentials are not sufficient to elicit a peptidergic response (repetitive activity is required; but see Whim and Lloyd, 1994), (b) peptidergic transmission can close, as well as open, voltage-dependent ion channels (not simply open voltage-independent ion channels, as occurs during ionotropic transmission), (c) peptidergic transmission can have a long and variable onset latency, and it builds up and decays slowly (ionotropic transmission has a brief and constant onset latency, and it rises and falls relatively quickly), and (d) peptides can linger/have access to their receptor for a long time post-release, anticipating the subsequent discovery that neuropeptides are inactivated post-release by either local or distantly located peptidase-mediated cleavage.

An interesting side note of the associated electrophysiological experiments in Jan and Jan's 1982 paper was the use of a 'manual voltage clamp' to analyze peptide-elicited membrane currents. This manual approach – rarely, if ever, appearing in current publications – was possible because (a) the peptide-mediated depolarization was very slow and (b) the peptide-elicited currents were readily accessible to experimenter control, as they were generated across the somatic membrane (Jan and Jan, 1982). This enabled the Jans to use their intrasomatic recording electrode to continually counter-balance the slow, peptide-mediated membrane potential change (thus, clamping the voltage) by manually turning the current injection dial on the recording amplifier. The result of these manipulations lacked the quantitative precision of the modern day circuit-based voltage clamp, but nevertheless provided accurate insight into the extent to which peptide application mimicked the actions of peptidergic transmission.

There can be no doubt that the paper by Jan and Jan (1982) that is the subject of this article has had lasting impact. This is evident in the fact it has received approximately 340 citations since its publication. While this number of citations may not seem impressive by the standards of recent high-profile publications, the research community was considerably smaller in the 1980s. Moreover, this paper continues to be acknowledged today (in fact, 2016 was the first year since 1982 when it garnered no citations, but there will be at least two citations in 2017) as seminal to the now-burgeoning field of peptidergic (co)transmission, a process which is clearly integral to nervous system function in all animals (Nässel, 2009; van den Pol, 2012; Nusbaum et al., 2017).

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