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COMMENTARY

IS THERE FEEDBACK REGULATION OF NEUROTRANSMITTER RELEASE BY AUTORECEPTORS?

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"The great tragedy of science —— the slaying of a beautiful hypothesis by an ugly fact".
Thomas Henry Huxley

The idea that the quantity of neurotransmitter released at nerve terminals during nerve activity is modifiable through the action of lingering neurotransmitter from previous nerve activity is called the hypothesis of feedback regulation of neurosecretion. According to this hypothesis, transmitter release per impulse is either decreased or increased by the action of transmitter released earlier in time from the same or neighbouring release sites. The concept of feedback regulation introduces an overriding terminal regulatory event into the neurosecretory process. A defect in feedback, then, could account conceivably for a multiplicity of clinical conditions, ranging from hypertension to mental depression, in which a functional excess or deficiency in neurotransmitter concentration is postulated. Consequently, the theory building value of the hypothesis is considerable.

Although the presynaptic feedback hypothesis originated from a consideration of certain previously unexplained effects of noradrenaline and its antagonist, phenoxybenzamine, on the liberation of endogenous noradrenaline from sympathetically innervated systems in the periphery (see reviews, Refs. 1–3), it is invoked now to deal also with transmitter mechanisms in the central nervous system, and expanded from its original base in adrenergic mechanisms into a generalized statement about neurosecretory regulation. It is presumed applicable to cholinergic, adrenergic, dopaminergic and other secretory neurones in the peripheral and central nervous systems.

A well understood principle of experimental science, succinctly expressed by Popper [4], is that "the criterion of the scientific status of a theory is its falsifiability, or refutability, or testability". The hypothesis that the increases and decreases in transmitter release by antagonists and agonists are due to interactions with receptor sites mediating functional neuronal feedback needs to be put to the test, and its credibility assessed. This can be done only by setting out reasonable expectations for a functional neuronal feedback system which are then met or not met experimentally. It is essential that the data obtained be analyzed for congruence with theoretical expectations, set out clearly in advance of the experiments and not formulated retroactively. Some work on presynaptic receptor theory seems to suggest that non-compliant data can be dismissed *a posteriori* as idiosyncrasies of the particular feedback system under study rather than as indices of a fundamental discordancy between experimental observations and theory. This approach to discrepant data is not easily acceptable since it would put presynaptic theory beyond the realm of "falsifiability". Let me illustrate this by looking at the correspondence between theoretical prediction and experimental results which is seen when describing the effects of variations in stimulation pulse train length on the magnitude of antagonist enhancement of transmitter release.

One such study examined a dopaminergic system in rabbit striatal slices. It was decided in that work that the reason pulse train length and antagonist effect did not correlate predictably was that, with longer trains of pulses (360 pulses vs 30, 60 or 90 pulses), the presynaptic system must be "shut down" [5]. Consequently, the failure to observe a theoretically predicted effect simply indicated "an inappropriate choice of the stimulation parameters" rather than an inadequacy in the hypothesis. Another report dealt similarly with non-compliant data [6]. Contrary to theory, the presynaptic antagonist scopolamine enhanced the liberation of [^{3}H]acetylcholine in guinea pig myenteric plexus at 3 and 6 pulse train lengths, but not at the longer 10 or 180 pulses. Instead of questioning the tenets of feedback theory, the data were interpreted to mean that "with longer trains of stimulation (in this case, anything longer than 6 pulses), the negative feedback mechanism plays only a minor role in regulating the output per pulse". In contrast, an opposite discrepancy between theory and results in the rabbit ear artery was resolved by concluding that a "prerequisite for the release-enhancing effect appears to be a sufficient length of the pulse train", which was identified as between 20 and 52 pulses [7]. In other words, in that study, it was decided that with short pulse trains feedback does not operate. The more likely possibility in each of the above instances simply is that feedback theory, when put to the test, does not adequately explain what happens when antagonists act on neurones.

My purpose here is to consider the body of experi-

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mental evidence now available from a number of laboratories which indicates that the presynaptic effects of agonists and antagonists on transmitter release cannot be explained by presynaptic receptors mediating functional feedback, and to encourage the reader to consider alternate suggestions as to how these drugs might work. Hopefully, this will lead to further exploration of the mechanisms of action of drugs which interact with neuronal processes. This article will not consider in detail the literature on the origin and development of presynaptic receptor theory. The reader is referred to some excellent articles elsewhere which cogently summarize the evolution of the concept [1, 2].

Criteria to establish feedback regulation

It is my opinion that five distinct and testable criteria each need to be consistently met, with any exemptions fully justified, before we can conclude that in a given system the pulse to pulse output of neurotransmitter is set by the ambient perineuronal concentration of previously released transmitter. These criteria are described below as they relate specifically to inhibitory or negative feedback systems. Positive feedback systems differ in some distinct respects and reference to them will be made separately later.

1. Presynaptic receptor antagonists, by disengaging feedback, should increase transmitter output in direct proportion to the amount of interrupted autoinhibition. By this is meant that, under conditions where feedback inhibition is expected to be negligible, the antagonists should have little effect on output, and, where feedback activity is predicted to be substantial, their effect should be large. An all or none effect of the antagonist would represent feedback inihibition only in a severely limited form, simply a two-position switch which would be insensitive to perineuronal transmitter levels except under the transition condition. In practical terms, a feedback system which is capable of gradated performance, but is maximally activated at or near threshold conditions for the effector response also approaches a two-position on/off switch. A further necessity when relating antagonist effect to block of feedback is that several blocking agents need to be tested and the maximal magnitude of enhancement should not differ between them, so long as they have been established in advance to be effective antagonists at presynaptic receptors.
2. Presynaptic receptor agonists, when added exogenously, in particular the transmitter substance itself (since it is the mediator of the presumed auto-inhibition), should reduce neurotransmitter release in inverse proportion to the extent of ongoing auto-inhibition. This is because, with increasing neuronal activity, the presynaptic receptors become occupied to an increasing extent by endogenously liberated transmitter. Consequently, a fixed quantity of exogenous agonist becomes a progressively diminishing percentage of the total agonist incident on the receptors. Further, the profile of the decline in agonist-induced inhibition of neurosecretion with increasing intensity of stimulation should correlate inversely with the profile of antagonist enhancement of release, under similar test conditions, since both are presumed to derive from occupation of the identical preysnaptic sites by increasing amounts of endogenous transmitter.
3. The effector response (e.g. muscle, gland or another nerve) is the *raison d'être* of neural transmission and the incontrovertible index of physiological relevance. Consequently, during nerve stimulation of differing intensities, the magnitude of the antagonist effect on transmitter release, signifying blockade of ongoing auto-inhibition, should be meaningfully interpretable, in the physiological sense, in terms of the response characteristics of the system under study.
4. The stimulation frequency-transmitter output curve and other similar indices of increasing neuronal activity, measured over the physiological range, in the absence of drugs, should reflect the increasing activity of an auto-inhibitory feedback system. This means, in general terms, that the per pulse liberation of transmitter should decrease under conditions which raise the perineuronal transmitter density.
5. A concentration of antagonist which enhances fully transmitter liberation by occupation and blockade of sites through which the endogenous agonist (neurotransmitter) mediates auto-inhibition should simultaneously block completely the inhibitory effect of exogenous agonists including the neurotransmitter substance itself.

It is the purpose of my essay here to show that the presynaptic receptor hypothesis does not meet satisfactorily even one of the five criteria described above. Fulfillment of all five should ordinarily be obligatory to confirm the operation of a feedback system. Otherwise, the interaction of terminal neuronal elements with release facilitating and inhibiting drugs requires explanations involving mechanisms unrelated to feedback regulation. The emphasis in this report is on adrenergic mechanisms, since most of the research has been done in this area. The principles expressed here, however, apply just as well to other putative presynaptic regulatory systems, for which the existing positive evidence is still quite meager, consisting generally only of observations that one or two antagonists of the system under study increase stimulation-induced transmitter release whereas agonists do the opposite.

Presynaptic blockade and transmitter release

Within the operating range of any negative feedback system, the more intense the stimulation conditions, the more substantial should be the potentiating effects of presynaptic receptor blockade. This is because auto-inhibitory activity, by definition, increases with increasing intrasynaptic density of transmitter.

Early studies on the effects of antagonists on transmitter release used the haloalkylamine compounds, generally phenoxybenzamine, and they were usually studied at one moderate and one unphysiologically high frequency and with results which offer no par-

ticular substantiation for a system sensing the biophase level of free and active noradrenaline. For example, Bell and Vogt [8] observed that phenoxybenzamine enhanced output in a guinea pig artery at 5 Hz but not at 25 Hz and a similar relationship was reported with 2 and 50 Hz in rat vas deferens [9] and with 5 and 30 Hz in cat spleen [10]. Also, Brown and Gillespie [11] and Kirpekar and Cervoni [12] found that haloalkylamine antagonists enhanced output at 10 but not at 30 Hz in cat spleen. Hughes [13], in the rabbit vas deferens, reported that the haloalkylamine increased output to a similar extent when 240 pulses were given at either 2, 6 or 16 Hz. McCulloch *et al.* [14] noted that the presynaptic antagonist increased output to the same extent at 4 and 8 Hz in rabbit pulmonary artery.

Experimental conditions which can be utilized to raise the intra-synaptic level of neurotransmitter in a predictably graduated way include: (a) increasing the frequency of stimulation over the likely physiological range, and thereby reducing the interval available between pulses for peak extracellular transmitter concentrations to decline; (b) increasing the total number of pulses in the pulse train, achieving an increased total cumulation of transmitter in the synapse during a brief stimulation period; (c) increasing the voltage of stimulation from threshold to maximal and in this way gradually increasing primarily the recruitment of individual release sites and, consequently, the total amount released; and (d) lengthening the duration of individual stimulation pulses, at supramaximal voltage, and thereby increasing primarily the quantity of transmitter released from individual release sites. Specific interventions can also be made with respect to ion fluxes, which contribute to action potential duration or to neurosection, to alter the quantities of transmitter released with each impulse.

Increasing the stimulation frequency, pulse train number, voltage (below supramaximal) or pulse duration reliably increases the intra-synaptic concentration of transmitter, and this is confirmed by monitoring the size and contours of the postsynaptic responses. This has been done by my laboratory in a number of test systems, such as the vas deferens of guinea pig, the sphincter iris muscle and the radial and renal arteries of cattle, and is illustrated in Fig. 1. Modifications to pulse number and frequency, in particular, are the means through which the central nervous system seems to regulate efferent autonomic activity and, undoubtedly, a similar operation occurs within the central nervous system as well. However, in a wide number of preparations exposed to conditions designed to alter perineuronal transmitter density in a manner relevant to the effector response, the effects of antagonists do not appear interpretable in terms of extracellular transmitter dynamics [15–24].

In one study, heart, spleen, vas deferens, ureter, uterus, artery and iris muscle taken from five species, namely rat, guinea pig, rabbit, cattle and pig, were excited with field stimulation at 1, 2 and 5 Hz with 10, 50 and 200 pulses (Fig. 2; Kalsner, unpublished observations). Treatment with yohimbine, an acknowledged potent presynaptic antagonist, revealed variable patterns of enhanced release which bore no obvious relation to extracellular transmitter levels and did not coincide at all with theoretical expectations for blockade of an increasingly activated negative feedback system. In another approach, yohimbine increased the overflow of endogenous noradrenaline from the rat heart stimulated by the cardiac sympathetic nerve trunk, but did so to an equivalent extent at 1 Hz and 4 Hz [24]. These workers commented that "the functional significance of an auto-inhibition that operates to a

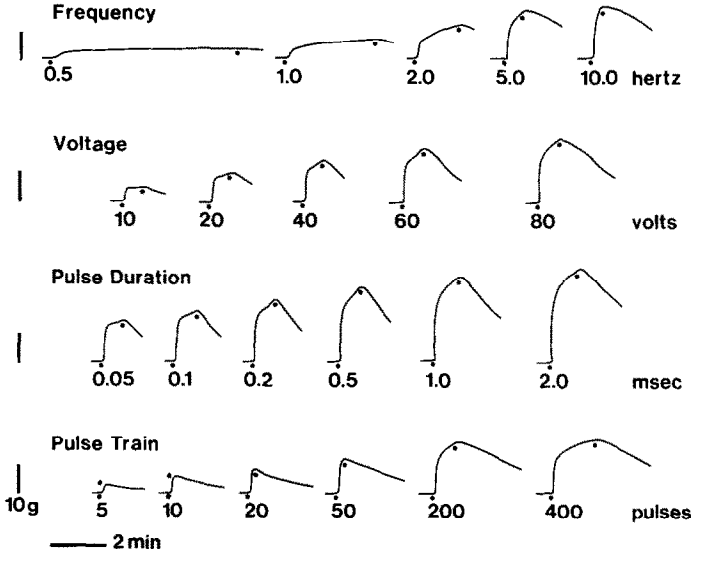

Fig. 1. Postsynaptic responses of cattle radial artery to progressive increases in the parameters of nerve stimulation. All vascular strips were superfused and stimulated through parallel platinum electrodes with 200 pulses, 4 Hz at 0.5 msec duration and 70 V except for the specifically indicated variable in each case. (a) Frequency varied between 0.5 and 10.0 Hz; (b) Voltage varied between 10 and 80 V; (c) Pulse duration varied between 0.05 and 2.0 msec; (d) Pulse train number varied between 5 and 400 pulses.

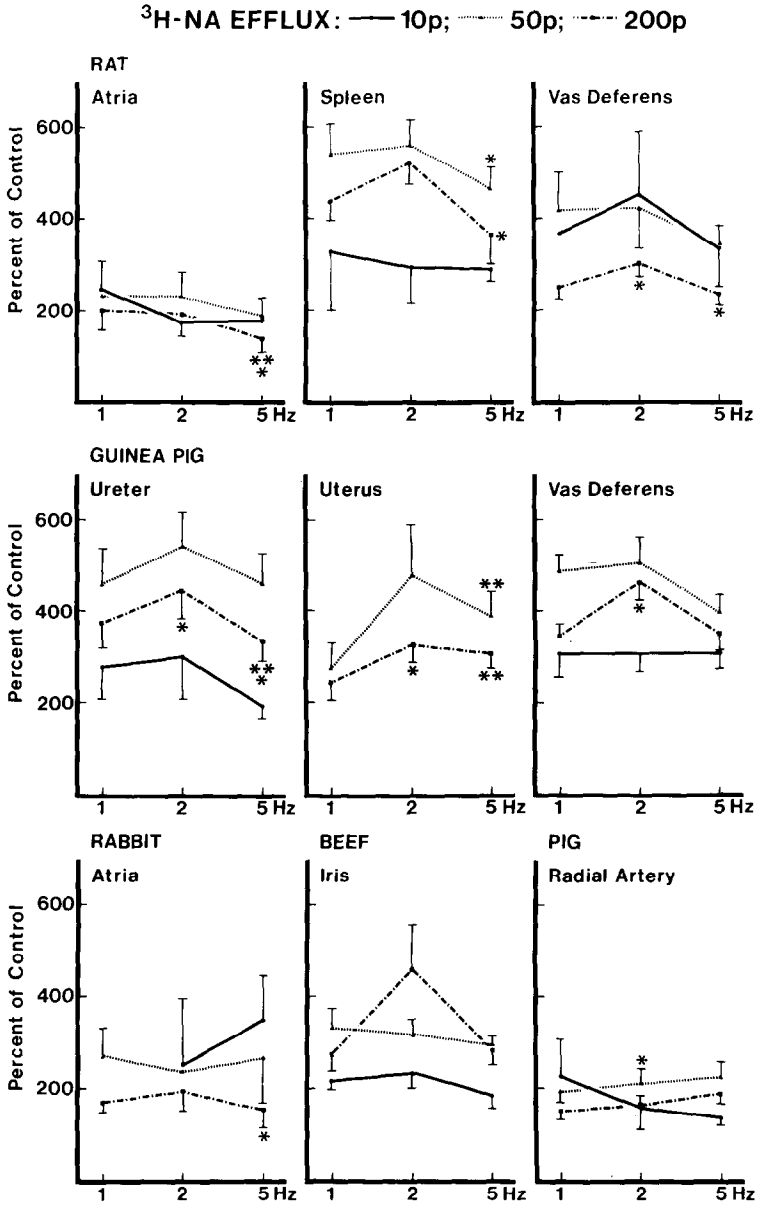

Fig. 2. Effect of increasing stimulation frequency on yohimbine enhancement of [^{3}H]noradrenaline output at three different pulse train lengths (10, 50 and 200 pulses) in nine tissues. Mean percentage values are shown with their standard errors and represent stimulation-induced output of tritium in yohimbine-treated groups as percent of output in matched control groups. Percentage values were obtained by comparison of individually determined output values during stimulation for paired yohimbine-treated and control tissues taken from the same animals. Number of values at each point averaged six. Key: (*) value significantly ($P < 0.05$) different from that at next lower frequency on the same curve; and (**) value at 5 Hz significantly ($P < 0.05$) different from that at 1 Hz on the same curve.

similar degree at both augmented and basal levels of neural activity is not clear". Other experiments with presynaptic antagonists, in several test systems *in vitro* [25], showed that, even with very broad frequency ranges (0.5 to 15.0 Hz) or prolonged pulse train lengths (up to 300 pulses) or, conversely, even with exceedingly brief pulse trains (4 pulses), results supportive of the feedback hypothesis were not forthcoming.

Discrepancies between theory and observation were also apparent when the amount of neurotransmitter released into the target tissue with each impulse was modulated by using a range of submaximal voltages [26]. Although the transmitter output with 300 pulses at 50 V in transmurally stimulated guinea pig ureters at 2 Hz was about ten times that at 10 V, the enhancement of release by yohimbine was essentially the same over the entire voltage range

(10–60 V), averaging about 300% above control values.

In another relevant approach, done with seven tissues, the amount of neurotransmitter liberated at sympathetic varicosities, by field stimulation, was altered progressively by increasing the duration of each stimulation pulse from 50 μsec to 2000 μsec with all other parameters of stimulation held constant. Prolonging depolarization produced the anticipated increases in transmitter release. The amount of transmitter liberated using 100 pulses of 2000-μsec duration averaged generally about five times that with 50-μsec duration pulses in most of the test preparations. The enhancement of transmitter release by yohimbine and also by the non-equilibrium haloalkylamine antagonist phenoxybenzamine, however, did not correlate positively, as theory predicted, with the relative amounts of transmitter in the synapse (Fig. 3). The presynaptic antagonists altered transmitter release most significantly when the pulse durations were shortest and the synaptic transmitter levels lowest. The blockers had their least effects at the longer pulse durations when extracellular transmitter levels were highest. Expressed in absolute terms, the effects of yohimbine and phenoxybenzamine appeared to be essentially "all or none". That is, the total amount of transmitter released during the 100 pulse cycle of stimulation, in the presence of the antagonist, approached the same value (in dpm) at each of the pulse durations. The insight this provides into the actual mechanism by which yohimbine raises transmitter output will be dealt with later in this commentary.

A similar lack of correspondence between per pulse output of transmitter, extracellular transmitter levels, and the quantitative effects of presynaptic antagonists was conspicuous when the extracellular calcium concentration was titrated (from 0.1 to 6.9 mM), to alter intracellular calcium flux at the nerve terminals, during neurosecretion [28].

The effects of antagonists in the central nervous system appear to be qualitatively similar to those already described for peripheral systems. In slices of rat occipital cortex or hypothalamus, the effects of yohimbine and phenoxybenzamine did not correlate well with frequency or pulse number changes (e.g. Table 1). The effects of the so-called presynaptic antagonists seem unrelated to interruption of a feedback system in the central nervous system just as they do in the periphery. Heepe and Starke [29], however, reported that, in rabbit cortical slices, presynaptic antagonists increase transmitter release more at 3 than at 1 Hz. These findings are in contrast to most other available work done within the physiological frequency range, and their observations appear unrelated to feedback blockade. They found enhancement at 1 Hz by the presynaptic antagonist rauwolscine to be about 350%, but by another antagonist, idazoxan, the value was about half that, namely 175%. Since the antagonist effect reflects simply occupation of a fixed population of presynaptic sites, the amount of enhancement by the two antagonists should have been identical.

Decay from peak transmitter concentrations at the synapse between impulses reflects biophysical considerations of transmitter dispersion, distri-

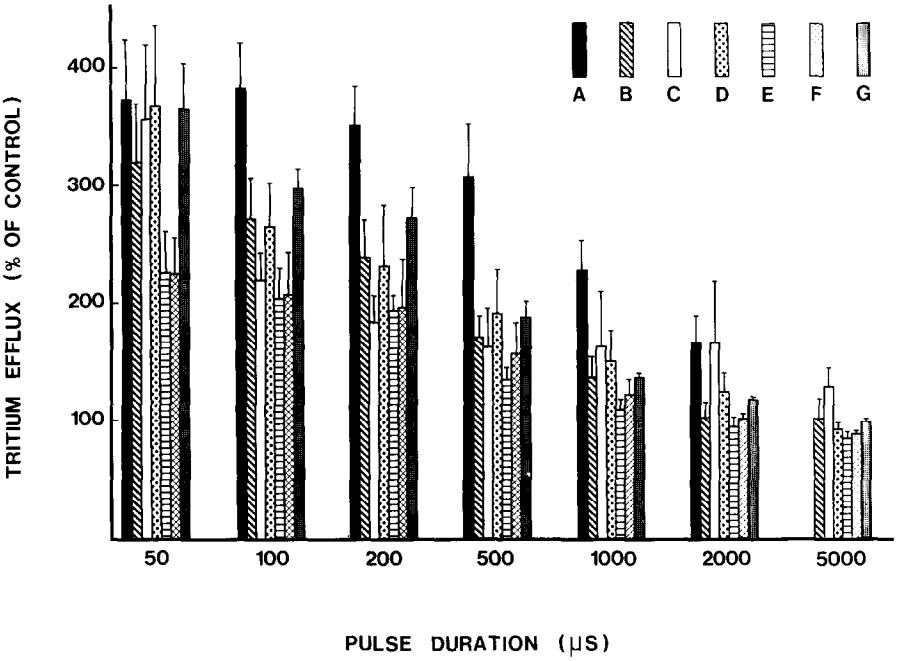

Fig. 3. Effect of yohimbine on stimulation-induced output of ^{3}H-transmitter with 100 pulses at a range of stimulation pulse durations (50–5000 μsec) in seven tissues. Columns shown with standard errors (vertical bars) represent stimulation-induced output in yohimbine-treated groups as a percentage of output in matched control groups. Percentage values obtained by comparison of individually determined tritium output values for paired yohimbine-treated and control tissues were taken from the same animals. Symbols represent: guinea pig atria (A), vas deferens (B), and ureter (C); rabbit atria (D), and central ear artery (E); rat atria (F), and spleen (G). (Reprinted from the *Br. J. Pharmac.*, see Ref. 27).

Table 1. Effect of yohimbine on stimulation-induced [^{3}H]noradrenaline efflux in superfused rat occipital cortex slices

Experimental group	Stimulation frequency (Hz)	No. of pulses	No. of values	Transmitter efflux (dpm × 10^{-3}) First stimulation period	Efflux ratio (second stim. period: first period)	% of Control efflux*
(A)						
Control	0.5	100	9	15.52 ± 3.15	0.79 ± 0.07	
Yohimbine†	0.5	100	9	20.73 ± 3.93	2.64 ± 0.19‡	360 ± 43
Control	1.0	100	10	10.99 ± 1.97	0.79 ± 0.11	
Yohimbine	1.0	100	10	15.22 ± 2.60	2.56 ± 0.17‡	374 ± 45
Control	2.0	100	9	9.26 ± 1.69	0.88 ± 0.11	
Yohimbine	2.0	100	9	15.95 ± 2.87	2.14 ± 0.15‡	287 ± 55
Control	4.0	100	8	10.61 ± 1.74	0.77 ± 0.08	
Yohimbine	4.0	100	8	17.24 ± 4.51	1.77 ± 0.16‡	255 ± 42
(B)						
Control	0.5	50	6	11.97 ± 2.63	0.68 ± 0.07	
Yohimbine†	0.5	50	6	11.20 ± 2.45	2.13 ± 0.34‡	346 ± 82
Control	1.0	100	5	16.96 ± 4.47	0.74 ± 0.06	
Yohimbine	1.0	100	5	11.84 ± 4.29	2.94 ± 0.61‡	426 ± 126
Control	2.0	200	6	21.75 ± 5.18	0.60 ± 0.04	
Yohimbine	2.0	200	6	17.83 ± 3.90	1.96 ± 0.23‡	333 ± 45
Control	4.0	400	4	33.38 ± 7.78	0.58 ± 0.07	
Yohimbine	4.0	400	4	35.48 ± 7.53	1.69 ± 0.23‡	296 ± 41

(A) and (B) show the effect of yohimbine on stimulation-induced output of [^{3}H]noradrenaline in cortical slices with frequency and pulse train variations. Tissues were pretreated with cocaine and normetanephrine.

* Obtained by comparison of individually determined ratios for control and treated preparations taken from the same occipital cortex.

† Yohimbine (1×10^{-6} M), when given, was administered in the interval between first and second stimulation sets and maintained in the superfusate throughout S_2. One of each set of slices served as an untreated control.

‡ $P < 0.05$, compared with the ratio for the corresponding control group.

bution, and diffusion within and out of restricted spaces. Accordingly, the size of the antagonist-induced enhancement of output was postulated to be inversely proportional to the dimensions of the neuro-effector gap [30, 31]. It is now appreciated that, contrary to expectations of feedback theory, there is no obvious correlation between the magnitude of antagonist enhancement and the size of the synaptic gap, or the density of transmitter innervation or the pattern of nerve fiber termination [17, 32]. In most systems, whether it be autonomic effector, ganglionic synapse or cerebral cortical slice, transmitter output is generally doubled or tripled by yohimbine or phenoxybenzamine under almost all test conditions and variations do not seem explainable in terms of transmitter dynamics in the synapse.

There is no uniformity to the effects of a group of antagonists on transmitter efflux in adrenergic systems, and this is another telling point against adrenergic presynaptic receptor theory. Some alpha receptor antagonists decrease rather than increase transmitter release, some others have no substantial effects at all, and still others show a concentration-dependent biphasic pattern of inhibition and enhancement of release. This was evident in guinea pig atria and in cattle radial and renal artery preparations, which have all been studied in considerable detail [17, 25]. In the guinea pig tissue, phenoxybenzamine and dihydroergotamine enhanced stimulation-induced transmitter output over a wide (100-fold) concentration range, but the effects of chlorpromazine, tolazoline, phentolamine, and yohimbine were complex and essentially biphasic.

In quantitative terms, the maximum magnitude of enhancement of stimulation-induced transmitter output differed with the particular antagonist used, indicating that occupation of a fixed population of presynaptic sites is simply not an adequate explanation of their actions. In guinea pig atria, yohimbine, phentolamine, dihydroergotamine, phenoxybenzamine, tolazoline and chlorpromazine increased stimulation-induced output by 61, 111, 57, 82, 18, and 32% above control values respectively [17]. Prazosin, used by a number of investigators to represent purely postsynaptic ($alpha_1$) antagonism, inhibited transmitter output in atria, as if it were a presynaptic agonist. Garcia *et al.* [33] had earlier observed that phenoxybenzamine enhances potassium-induced efflux of [^{3}H]noradrenaline by 50% in cat spleen, but phentolamine at 10^{-7} to 10^{-5} M has no significant effect on output and, at higher concentrations, it severely depresses transmitter output. Similar discrepancies in the comparative enhancement of transmitter release by antagonists are apparent in the findings of Farnebo and Hamberger [34], Borowski *et al.* [35], and Enero and Langer [36]. Kalsner and

Chan [37], Angus and Lew [38] and Heepe and Starke [29] also noted that the antagonist phentolamine can inhibit, as well as enhance, transmitter release. In a novel neurobiological approach to the identity of presynaptic receptors mediating autoinhibition, Laduron [39, 40] was not able to find biochemical support for their axonal transport to nerve terminals, although the presence of heteroreceptors, such as those for acetylcholine, was confirmed. Laduron commented that "there is absolutely no evidence for an axoplasmic transport of autoreceptors". He questioned their identity and concluded that "these negative results may be regarded as an additional and more direct support to the idea that noradrenaline cannot regulate its own release through a negative feedback mechanism involving adrenergic-α_2 autoreceptors".

Transmitter liberated by a single stimulation pulse, by definition, is not subject to feedback, yet phenoxybenzamine, the antagonist initially employed to establish the feedback hypothesis, increased both the efflux of tritium (219%) and the effector contraction (300%) in response to one isolated pulse of 1-msec duration in guinea pig vas deferens [41]. The possibility that noradrenaline released early during such a brief event inhibits subsequence release by that same single pulse need not be taken seriously. The inward movement of calcium occurs during depolarization, as does the rise in intracellular "active" calcium, and these events are essentially ended with the end of the action potential [42]. The release of transmitter, however, lags behind the initiating depolarization starting after the end of a pulse of less than 5-msec duration [43].

The later of the two phases of calcium entry, occurring during the neuronal action potential, is most relevant to adrenergic transmitter release [44]. It turns on at the same time as the increase in potassium permeability [45], ant depolarization has a dual action serving both to activate and inactivate calcium entry. Rahamimoff [46] asserts that "applying calcium immediately after the depolarizing pulse does not produce any release of transmitter. Such experiments show that calcium action is very rapid on a biological time scale, and that it must be present either before or during the depolarization. It is ineffective if applied after the depolarization, even before the onset of transmitter liberation, that is, during the synaptic delay". It appears, then, that membrane events, as well as the intraneuronal increase in "active" calcium relevant to release, are over at the time of transmitter liberation. The finding of antagonist sensitization of release by a single pulse and of a magnitude comparable to that seen with any larger number of pulses and at any test frequency weighs heavily against the hypothesis. It is clear from this discussion that, when the first of the five criteria described above is considered, the evidence fails to root the quantitative aspects of antagonist enhancement in variations in the intra-synaptic density of transmitter.

Agonists and transmitter inhibition

Inhibition of stimulation-induced transmitter release by added noradrenaline or by some other agonist provides, in itself, no assurance that a functional feedback system is at work. Any one of a variety of agents with a bewildering array of actions can inhibit release. An inverse association between the synaptic concentration of neurally released transmitter and the inhibitory effect of an exogenous agent, over a reasonable stimulation range, must be demonstrable for the finding to be supportive of auto-inhibition. In the adrenergic system, for example, if feedback is operative, the two sources of noradrenaline locally released and externally provided would be co-contributors to a common pool of extracellular stimulant activating neuronal autoinhibitory receptors. Consequently, a fixed quantity of exogenous agonist should have a declining effect on output in the presence of a progressively increasing level of endogenously released transmitter as the stimulation conditions are intensified. This heightened neural activity can be achieved experimentally by altering any one of several stimulation parameters such as frequency or pulse train number or voltage. These procedures have been described above in the studies with antagonists.

Although a number of preparations have been examined, a correlation between intensity of nerve activity, over the physiological range, and agonist-induced inhibition has not been revealed [16, 47]. For example, in guineg pig atria, noradrenaline inhibited transmitter efflux by 80% at 0.5, 1 and 2 Hz and, in vas deferens, the effect of noradrenaline was the same, an inhibition of about 60%, over the broad stimulation range of 0.5 to 10 Hz. The distinctly different profile of effect for noradrenaline and for the antagonist with frequency and with train length indicated that they do not have a common site of action, and this is discussed further below.

From another vantage point, it was noted that noradrenaline inhibited transmitter release in guinea pig ureter to an equivalent extent, about 70%, at 20, 40 and 60 V although the absolute output of transmitter varied more than 3-fold with these voltage changes. A similar disparity between observation and theory in the inhibitory effect of exogenous noradrenaline was observed when major modifications were made to the extracellular calcium level and, accordingly, to the amount of neurotransmitter released with each impulse [28].

It should be appreciated that a stepped decrease in transmitter output by noradrenaline with accelerated neuronal activity is occasionally seen, but that it may not derive from the operation of an ongoing autoinhibitory feedback system. For example, dopamine (3×10^{-7} and 3×10^{-6} M) inhibits the stimulation-induced efflux of [^{3}H]noradrenaline from cattle renal arteries and does so to a declining extent with increasing frequency [1–15 Hz) [15], but no negative feedback loop operates for dopamine in this preparation: known antagonists of dopamine action, pimozide and metoclopromide, although antagonizing dopamine inhibition did not, by themselves, enhance stimulation-induced transmitter efflux nor did they block the inhibiting effects of exogenous noradrenaline on efflux.

Transmitter output and response site

It is not possible, ordinarily, to determine the postsynaptic consequences of antagonist-induced

enhancement of transmitter output (presumed due to presynaptic α-receptor blockade), because autonomic effector responses are usually mediated by α-receptors and these are also vulnerable to those very agents used to achieve presynaptic antagonism. One way around this problem is to seek out systems that have their responses mediated by an alternate receptor, namely the β-adrenoceptor. In one such study with cattle iris muscle, which responds with relaxation to sympathetic nerve stimulation, after treatment with atropine, yohimbine doubled transmitter output over a range of pulse train lengths varying from 10 to 100, but the β-receptor mediated smooth muscle relaxations were increased insignificantly by such an enhancement of transmitter liberation [48].

A similar discrepancy between presynaptic effects on release and the postsynaptic response was noted by Angus *et al.* [49] in their study of heart rate in guinea pig atria. They found no evidence for a role of auto-inhibition in modulating transmitter liberation with stimulation parameters that produced 50% of maximal tissue response and concluded, based on the amount of antagonist-induced enhancement, that "stimulation sufficient to induce a near maximal response" is necessary to evoke auto-inhibitory feedback. They go on to state that "auto-inhibitory feedback as a homeostatic mechanism of regulating neurotransmitter concentration would appear to be a last-resort mechanism".

Such findings are not surprising since effectors often require 3000-fold or more increments in environmental transmitter concentration to move from minimal to maximal responses, and increasing the amount of transmitter liberation by 50–100% would not be expected to alter response size in a major way. For example, in a vascular preparation which gives a threshold contraction to noradrenaline at 1×10^{-9}, a moderate contraction at 3×10^{-8}, and a near maximal response at 1×10^{-6}, an increase in the biophase concentration from 2.0×10^{-7} to 3.0, or even 4.0×10^{-7} would not yield a substantially improved response. This point is particularly obvious with respect to β-receptor mediated presynaptic positive feedback systems, where antagonist-induced changes in transmitter release in the order of only 10 or 20% are seen [50, 51]. Obviously, insubstantial postsynaptic consequences would be anticipated in such a system, if indeed the antagonist-induced effects were due to interaction with a feedback system.

The physiological impact of presynaptic receptor blockade was also looked at by Starke and associates using yohimbine which "enhances the overflow of tritium and the smooth muscle contraction induced by transmural sympathetic nerve stimulation" (α-receptor mediated) [52]. They showed that, although the maximal increment in ^{3}H-transmitter liberation at 2 Hz was 161% above controls, the contractions were increased very slightly, by a maximum of 20%. Additionally, at 4 Hz, an increase in tritium efflux of 109% was accompanied by only a meager 11% increase in the postsynaptic response. In still another study, it was concluded, in support of presynaptic theory, that phentolamine increased substantially both transmitter efflux and the chronotropic response to sympathetic nerve stimulation in guinea pig atria [53]. But different conditions were used to study the two variables and, consequently, the results are open to interpretation. For example, transmitter release was examined with 240 pulses at 5 Hz during an early phase of the experimental sequence, whereas heart rate was studied separately with five pulses at 0.5 Hz during a later phase, and the decline from peak antagonist effect, after washout, was not comparable in the two conditions.

Modifications of heart rate or blood pressure by presynaptic receptor antagonists have been examined to assess the operation of feedback *in vivo*. These studies have been done without the associated analyses of transmitter efflux, and with conflicting results [19, 21, 24, 54–56]. In one of them, done in the pithed rat, Drew and colleagues found that phentolamine had only very modest effects on heart rate during sympathetic nerve stimulation. The presynaptic antagonist increased the cardiac positive chronotropic response to sympathetic nerve activation at 0.2, 0.5 and 1 Hz by 21, 12 and 0% respectively. This pattern is precisely opposite to theoretical expectations, and Drew accordingly concluded that "the physiological importance of the presynaptic α-inhibitory feedback system in the regulation of end-organ responses, rather than noradrenaline release, remains unclear". In another study, unsupportive of auto-regulation, Dart and co-workers [24] found that yohimbine did not change significantly the chronotropic response to sympathetic nerve stimulation in the rat although transmitter output was approximately doubled.

Per pulse transmitter output

By definition, an operative negative feedback system demands that the per pulse output of neurotransmitter decrease with increasing neural activity because the increasing concentration of transmitter in the synaptic space increases auto-inhibition. Surprisingly, even this elementary requirement of presynaptic theory is not met when a variety of test preparations are examined under a range of stimulation conditions which alter extracellular transmitter density. In cattle vascular tissue and iris muscle or guinea pig atria and vas deferens, the expected correlations between frequency and per pulse output o. total pulse train number and per pulse output were not found [17]. For example, in vas deferens, the per pulse output of [^{3}H]noradrenaline did not vary with a 5-fold increment in pulse number, and it increased rather than decreased over the span of 0.5 to 15 Hz. A similar lack of correlation was seen when slices of rat cerebral cortex were examined (Kalsner, unpublished observations). One detailed study involving nine peripheral tissues is presented in Fig. 4, and the overall lack of concordance between theory and data is obvious.

The failure of per pulse transmitter output to decline reliably with increasing intensity of stimulation is a formidable obstacle to the viability of feedback theory particularly since these observations can be made in the absence of drugs, to which masking or competing actions or effects might otherwise be assigned. If one were to postulate that a competing process of facilitation of neurosecretion

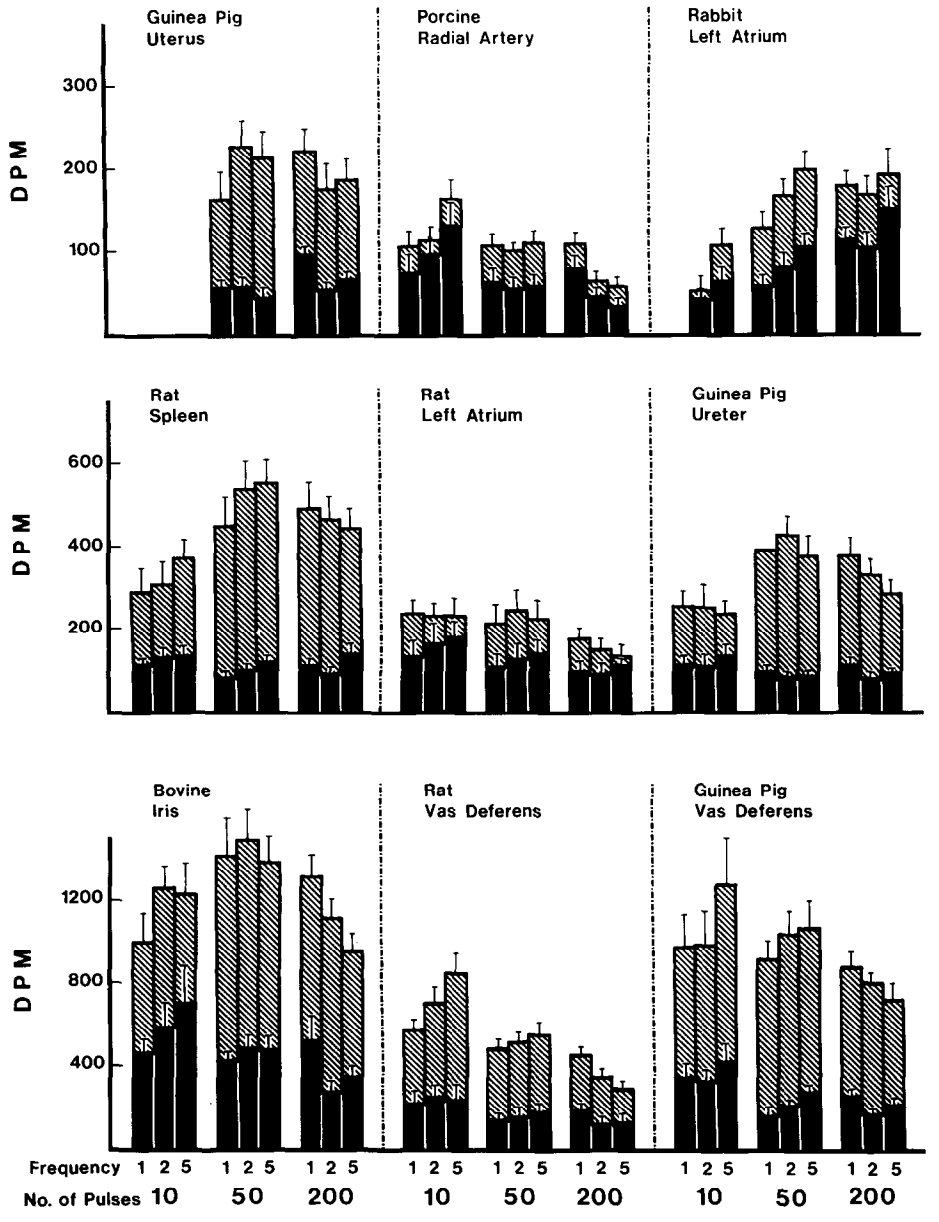

Fig. 4. Relationship between stimulation frequency and per pulse output of [^{3}H]noradrenaline in nine tissues in the absence and presence of yohimbine (3×10^{-6} M). Each tissue was stimulated with 10, 50 and 200 pulses at each of the three indicated frequencies. Per pulse output of ^{3}H-transmitter (dpm) is shown in untreated (solid bars) and yohimbine-treated (hatched bars) preparations. Mean values, of approximately six preparations in each category, are shown with their S.E.M.

masked the visibility of auto-inhibition, then feedback inhibition becomes reduced, at best, to the status of an accessory modifier of a primary process of facilitation. But even this does not appear to be so (Fig. 4). The presence of facilitation, masking the visibility of auto-inhibition, can be sought out with the aid of presynaptic receptor antagonists. Disconnection of negative feedback by presynaptic receptor blockade should potentiate facilitation by removing a countervailing inhibitory system. No such release from auto-inhibition was noted (Ref. 17 and Fig. 4). The change in per pulse output with increasing frequency or pulse number, in most cases, was not more steep in the presence than in the absence of a presynaptic receptor antagonist. Blakeley and colleagues [22] also failed to obtain support of feedback inhibition of per pulse output in their ingenious electrophysiological approach. Looking at the release of transmitter from single sites in the rat vas deferens, in the absence of drugs, they found "no evidence of any inhibition of release at the same release site from 500 ms to 3 s after release, and also no evidence of inhibition of nearby sites from 7 ms after release".

Agonists and antagonists

Enhancement of transmitter release by antagonists and their blockade of agonist-induced inhibition of release should be inseparable events according to presynaptic theory since both result from antagonist occupancy of presynaptic receptor sites. Early workers did not study in any precise way the relation between antagonist and agonist effect, and the concentrations of antagonist used by them may have been too high to allow separation of multiple presynaptic effects. For example, Starke [57] used 1 μg/ml of phenoxybenzamine to show that noradrenaline did not inhibit transmitter output after presynaptic blockade in the rabbit heart. Additionally, Starke and colleagues [58, 59] reported that phentolamine "shifted the presynaptic dose–response curves for noradrenaline and oxymetazoline to the right" in rabbit pulmonary artery, but they used an extremely high antagonist concentration (1×10^{-5} M). More recently, however, it has been shown that yohimbine, when used in the lowest concentration which gives maximal enhancement of release (3×10^{-7} M), does not substantially reduce the inhibitory effects of noradrenaline or of the imidazoline derivative, oxymetazoline, in guinea pig ureter [60].

Clonidine is widely considered to be the prototypical presynaptic receptor agonist. This imidazoline compound, however, has been reported to increase [61], decrease [62] or to have no effect on the liberation of noradrenaline from nerves. Such observations have been dealt with by some investigators by classifying clonidine as a partial agonist [62, 63], seemingly permitting a variety of contradictory effects to be assimilated within the context of presynaptic theory. Ariens *et al.* [64] have emphasized, however, that partial agonists exhibit fully predictable effects in the presence of full agonists such as noradrenaline or antagonists such as yohimbine and phenoxybenzamine, and these expectations need to be applied to clonidine. When this is done, it is obvious that a dualistic agonist/antagonist action through presynaptic alpha$_2$ receptors does not adequately account for the presynaptic effects of clonidine.

Baker *et al.* [65], for example, reported that yohimbine does not effectively antagonize the inhibitory effects of clonidine or of noradrenaline in dog saphenous vein in concentrations which enhance transmitter release and, in a study with guinea pig heart, a complex pattern of effect was revealed for clonidine which is not understandable in terms of partial agonist theory [66]. Clonidine, in a low concentration, inhibited release of [^{3}H]noradrenaline from field-stimulated cardiac tissue, but a higher concentration did not inhibit release. This is not in keeping with the concentration–effect profile of a partial agonist. The inhibitory effect of a partial agonist on transmitter release should, with increasing concentration, move towards, and then sustain, a plateau value equivalent to the maximal effect achieveable with the compound's limited intrinsic activity (E_{B_m}). Instead, a loss of efficacy with increased concentration was observed.

Further, modest inhibition of transmitter release by the lowest effective concentrations of clonidine in the experiments with cardiac tissue should have been blocked by yohimbine, and the effects of more substantial agonist concentrations attenuated. Instead, yohimbine did not at all antagonize the inhibitory effects of the agonist. In fact, clonidine, at one concentration (1×10^{-6} M), was more effective in inhibiting release with yohimbine present than absent. According to receptor theory and the dynamics of competition for a single occupation site, the agonist cannot produce a greater maximal response (E_{B_m}) in the presence of antagonist than in its absence. Korner and co-workers [67] similarly concluded from studies of the chronotropic response in field-stimulated guinea pig cardiac tissue that "clonidine has a concentration-related inhibitory effect on neural release of noradrenaline that is not related to any competitive effect for the presynaptic α-receptor between the drug and released noradrenaline".

Actions of clonidine unrelated to activation of presynaptic α_2-receptors have been identified (e.g. histamine-like and opiate-related) [17, 68–70], and these may explain some of the effects of clonidine currently attributed to activation of presynaptic adrenergic receptors.

Positive feedback

Some workers have proposed that positive feedback systems also function in the regulation of neurosecretion. To assess the theoretical feasibility of such auto-regulation, some general points about control systems need to be made. Feedback is a "method of controlling a system by reinserting into it the results of its past performance" [71]. It is necessary in a feedback system, in our case neurotransmitter output, that the feedback signal be transmitted from the output side of the system back to the input side, forming a closed loop. To establish a negative feedback, once a signal travels around the loop, its sign must be reversed. A negative feedback system is an "error actuated" system [72] which "tends to maintain a prescribed relationship of one system variable to another by comparing functions of these variables and using the difference as a means of control" [73]. Importantly, the deviation signal formed as a result of feedback is "used to control the action of a final control element in such a way as to tend to reduce the deviation to zero".

A closed loop without the reversal of sign, namely positive feedback, is not error actuated. It is unstable and creates a "vicious circle" [74]. In neurotransmitter terms, an output of transmitter sufficient to activate a barely threshold number of, for example, adrenergic beta receptors will initiate an unbraked feedback acceleration of per pulse output, regardless of frequency, which will continue unabated with each additional stimulation pulse. Output will increase until stopped by saturation of the receptor system or of an equivalent component. Thus, the desired behaviour is not compared with the actual behaviour and the difference used to constrain the actual behaviour. It is essential therefore to recognize the likelihood that, if beta or other supposed feedback sites do function under ordinary conditions of neurotransmission, they would be fully activated and the positive feedback system fully

engaged regardless of stimulation frequency, or instead they would be routinely nulled by the intervention of a negative feedback system. No obvious biological advantage accrues specifically to such an "all or none" system, when in fact, predictable and reliable graduations in response of an end-organ are desirable.

With respect to adrenergic mechanisms, it has been proposed that under conditions of low frequency stimulation, positive feedback via β-receptors assists neurotransmission by enhancing quantal release and consequently the size of the effector response, but that under more vigorous stimulation, the predominant α-inhibitory receptors are involved in mitigation of transmitter release and the concomitant response.

It should be noted, however, that α- and β-receptor systems are both simultaneously activated over a similar frequency range by transmitter noradrenaline as determined by considerations of receptor-agonist affinities and by the maximal and minimal effects of exogeneous agonist [51]. For example, the maximal inhibitory effect of noradrenaline and the maximal enhancing effect of isoprenaline both occur at 1 Hz (e.g. guinea pig atria [51]; cat spleen [2, 53]). Similarly, both agonists show minimal effects at identical frequencies [2]. It should be appreciated that the relative effectiveness of the exogenous agonist is inversely related to the degree of presynaptic activation by released transmitter under the experimental range of test conditions.

The critical evidence which is purported to verify the existence of excitatory presynaptic β-receptors mediating positive feedback is that the β-stimulant isoprenaline enhances the stimulation-induced output of ^{3}H-transmitter in several test preparations (e.g. Refs. 10, 58 and 75) and that propranolol blocks this effect in cat spleen [76]. However, some workers [77–79] have failed to observe an effect of isoprenaline on the stimulation-induced tritium efflux in a number of tissues, such as rabbit pulmonary artery, mouse atria and rat cerebral cortex, raising doubts as to the general applicability of the earlier positive findings.

Work from my laboratory has revealed that the presynaptic sites for isoproterenol do not show the necessary stereospecificity of beta receptors and that a "nonspecific" action of propanolol, shared by the *d* and *l* isomers, may account for its antagonism of the isoproterenol effect [51]. Further, in another study done in guinea pig atria, the quantitative enhancement of efflux by isoproterenol was found to be of similar magnitude at three of the four test frequencies, suggesting no direct competition with increasing perineuronal levels of endogenous transmitter in the activation of receptor sites. A number of other objections to the universality and validity of the hypothesis of presynaptic beta receptors have been enunciated elsewhere [1, 77]. A more reasonable suggestion has been that adrenaline released from the adrenals and circulating in the blood may increase transmitter release under certain conditions by a presynaptic action [80, 81]. Although such a system is theoretically feasible, it does not represent positive feedback of transmitter release and remains to be convincingly demonstrated.

Summary

Neurotransmitter release does not seem to be regulated by neuronal receptors mediating feedback and the mechanism of action of presynaptically active agents is still uncertain.

In a recent set of papers [27, 82], experiments were described in which major modifications were made to the amount of neurotransmitter released per impulse, with all other parameters of field stimulation, such as pulse number, voltage and frequency, fully controlled. These studies done with a number of sympathetically innervated tissues give some insight into an antagonist action presynaptically which is independent of the ambient concentration of extracellular transmitter. It appears to involve, instead, the gating mechanisms which control neuronal membrane depolarization and repolarization. It was found that the effects of yohimbine and also of phenoxybenzamine on stimulation-induced efflux appeared to be essentially "all or none". That is, the absolute total release of tritiated transmitter with 100 pulses was elevated to roughly the same dpm value by the presynaptic antagonist at each of the pulse durations between 50 and 1000 μsec, in a variety of test tissues. The declining percentage effect of the antagonist on tritium efflux, as the pulse duration was enlarged between 50 and 1000 μsec, referred to earlier (Fig. 3), was due to rising values for transmitter release in the controls not matched by proportionally similar increases in the antagonist-treated tissues.

Values for the amount of transmitter released during stimulation in the presence of yohimbine, at pulse lengths between 50 and 1000 μsec, were all in the range of values achieved in the absence of yohimbine with long pulse lengths (1000–2000 μsec). In other words, prolongation of the pulse duration from 50 to 1000 μsec and the exposure of tissues to a presynaptic antagonist, such as yohimbine or phenoxybenzamine, may involve a common mechanism, and the effects of these two procedures are not additive. In fact, with much prolonged pulse durations (2000–5000 μsec), the presynaptic antagonists are virtually ineffective.

It is known that the release of transmitter from sympathetic nerves is directly related to the duration of the action potential. If it is prolonged, the calcium channels stay open longer leading to greater entry of calcium and to an increased release of transmitter [45, 46]. Yohimbine and phenoxybenzamine may prolong the duration of depolarization by indirect modification of the calcium gating mechanism.

Repolarization begins with the turning on of the potassium current, and the whole of the maintained outward current appears due to an efflux of potassium ions [83, 84]. This process may be interfered with by presynaptically active agents. Tetraethylammonium (TEA), one of the few substances known to selectively block outward potassium permeability directly [84, 85], prolongs the duration of the action potential and, hence, neurosecretion [27, 72]. The quaternary ion increased transmitter release in a way somewhat like yohimbine, although the values reached were higher and the similarity not complete [27]. TEA can disrupt entirely neuronal function

leading to repetitive action potentials and discharge of transmitter stores [86].

Above all, the primary purpose of this essay is to encourage more investigation into the very basis of the evidence used to structure existing presynaptic theory. The body of material reviewed here raises serious questions about the idea that the presynaptic effects of agonists and antagonists are related exclusively, or predominantly, to interaction with neuronal systems sensing and responding to fluctuations in extra-neuronal transmitter levels. These concerns hold true, not only for adrenergic mechanisms, where the case is strongest, but also for systems involving other transmitter substances, both in the periphery and in the central nervous system, for which little positive evidence has been provided. Although an alternative hypothesis has been offered here in a bare and rudimentary form, other avenues need to be explored. In the end, no unifying hypothesis encompassing all the experimental observations may be available. Whatever finally emerges may prove less theoretically satisfying, even if more accurate.

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