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COMMENTARY

UNEXPECTED PLASTICITY AT AUTONOMIC JUNCTIONS

ENVIRONMENTAL REGULATION OF NEUROTRANSMITTER PHENOTYPE AND RECEPTOR EXPRESSION

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During the past decade, our view of synaptic transmission at autonomic junctions has been altered significantly. First, the notion that postganglionic neurons use one of only two transmitters, norepinephrine and acetylcholine, has been revised; there are additional small molecule transmitters such as serotonin [1–3] and the purines [4–7] as well as an ever increasing number of neuropeptides [8, 9]. Although initial colocalization studies provided evidence for preferential associations of particular small molecule transmitters and neuropeptides in autonomic neurons, for example acetylcholine with vasoactive intestinal peptide and norepinephrine with neuropeptide Y, many different combinations are, in fact, used [10–12]. Further, analysis of neuropeptide actions in several autonomic target tissues has revealed an unexpected complexity of pre- and postsynaptic effects that may be more long-lasting than those of the small molecule transmitters [13, 14].

Not only has the number of putative neurotransmitters increased, but so has the number of receptor subtypes for many of the transmitter candidates. For example, based on pharmacological criteria, affinities for selective antagonists, muscarinic receptors were divided into two and then three subtypes [15–17]. Recently, molecular cloning studies have shown that the muscarinic receptors constitute a gene family of not just three, but five, closely related polypeptides [18–23] possessing distinct functional properties [24–26] and distributions [20–27]. Molecular heterogeneity of adrenergic receptors also has been uncovered, although the functional consequences are not yet as well defined [28–35]. Since muscarinic, adrenergic and many neuropeptide receptors are coupled to intracellular effector proteins via guanine nucleotide regulatory proteins (G-proteins), cellular responsiveness requires not only the presence of the receptor but also a functional transduction system. The G-proteins are heterotrimetric proteins consisting of an α-, β-, and γ-subunit; four broad classes are defined on the basis of the α-subunit [34–37]. The α-subunit is thought to interact directly with the receptor, while the β- and γ-subunits are generally believed to act in a structural capacity, although recent evidence suggests that these subunits may play a physiologic role as well [38]. As in the case of the transmitter receptors, each G-protein subunit is represented by a gene family; molecular cloning techniques have identified 12 unique α-, 2 β-, and 3 γ-subunits [35]. Thus, the potential exists for the control of cellular responsiveness in autonomic target tissues by regulating the expression of the multiple receptor subtypes and/or the expression of the signal transduction proteins.

In addition to the proliferation of accepted neurotransmitters and receptors, our view of autonomic transmission has been altered by mounting evidence that neurochemical plasticity is part of the normal repertoire of the peripheral autonomic nervous system. It has long been recognized that quantitative changes occur presynaptically through regulation of transmitter content in response to altered levels of activity and of trophic factors, and postsynaptically through regulation of transmitter receptor number in response to altered levels of transmitters or hormones [39–42]. Such changes could obviously influence the efficacy of synpatic transmission at particular junctions. It is now clear that qualitative changes can occur as well, and it is these which are the topic of the present review. The existence of plasticity has been most clearly established for transmitter properties of sympthetic neurons; as described below, they can change their classical transmitter and alter their neuropeptide expression. Although transmitter plasticity was first discovered in cell culture studies, examination of development *in vivo* indicates that the plasticity observed *in vitro* is part of the normal history of some sympathetic neurons and possibly of many neuron classes. An unexpected finding is that environmental cues play a crucial role. Regulation of transmitter receptor expression and responsiveness in autonomic target tissues is not as well understood as the determination of the transmitter phenotype, but in the several systems reviewed below evidence exists suggesting that environmental signals influence the expression of receptor as well as other components of the signal transduction system such as G-proteins.

Environmental regulation of transmitter choice in vitro

The role of environmental cues in determining the

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neurotransmitter choice of autonomic neurons was revealed in studies of dissociated sympathetic neurons developing in cell culture. At birth in the rat, all sympathetic neurons in the superior cervical ganglion (SCG) are noradrenergic [43–46]. When neurons are dissociated from the SCG and grown in defined medium or under conditions in which they are chronically depolarized, they continue to develop as noradrenergic neurons [47–50]. If, instead, the neurons are grown in the presence of certain types of non-neuronal cells or in medium conditioned by such cells, the neurons decrease their expression of noradrenergic properties and acquire cholinergic function [46, 51–55]. In the case of heart or skeletal muscle cells, the induction of cholinergic properties occurs through the release of a soluble cholinergic inducing activity. The effect of this conditioned medium or cholinergic differentiation factor is blocked when the growth conditions mimic increased neuronal activity, such as the presence of elevated potassium [47, 50]. Studies of neuron/heart microcultures in which the changing transmitter properties of single neurons were followed over time have shown unequivocally that the cholinergic factor produced by nonneuronal cells induces neurons that have already begun to differentiate along a noradrenergic pathway to become cholinergic [55–57].

Initially, studies of neurotransmitter choice in cultured sympathetic neurons focussed on the role of cholinergic differentiation factor from heart cell conditioned medium. This factor is a relatively heat-stable 45 kD glycoprotein [58, 59]; removal of the carbohydrate yields a 22 kD protein which retains biological activity. Antisera raised against a partial peptide sequence obtained for cholinergic differentiation factor both immunoprecipitate the 45 kD protein and the cholinergic inducing activity [60, 61]. Recently, the factor has been sequenced and found to be identical to a hematopoietic differentiation factor, Leukemia Inhibitory Factor. It is of interest that this factor causes the differentiation of certain kinds of myeloid cells as well as inducing cholinergic function in sympathetic neurons [62].

It is now clear, however, that the cholinergic differentiation factor is not the only factor able to induce cholinergic function in cultured sympathetic neurons; a number of more or less well characterized environmental signals have similar effects. Ciliary neurotrophic factor, originally identified because of its ability to support the survival of ciliary neurons in culture, has been found recently to induce choline acetyltransferase, the synthetic enzyme for acetylcholine, and to decrease tyrosine hydroxylase, the rate-limiting enzyme in the catecholamine synthetic pathway, in cultured rat sympathetic neurons [63]. Two membrane-associated molecules which induce cholinergic function in cultured rat sympathetic neurons have been partially purified recently from spinal cord [64–67]. In addition, human placental serum, rat serum, chick embryo extract and a heparin-binding activity in brain extract induce cholinergic function although they do not appear to decrease noradrenergic function [48, 68–71]. It remains to be determined whether these several cholinergic-inducing factors and activities are the same or different. Comparison of the biological and immunological properties of three of the best characterized factors, the cholinergic differentiation factor, the ciliary neurotrophic factor and MANS, one of the membrane-associated neurotransmitter-stimulating factors [66], indicates that the cholinergic differentiation factor is different from the ciliary neurotrophic factor and MANS [61]. This finding has been confirmed with the recent publication of the sequences of CNTF [72, 73] and CDF [60].

Environmental factors can influence the choice not only of traditional neurotransmitters by cultured sympathetic neurons but they also affect the expression of neuropeptides. For example, coculture with nonneuronal cells increases the expression of substance P, whereas growth in culture conditions which mimic activity decreases it [74, 75]. Co-culture with nonneuronal cells from different autonomic targets or in medium conditioned by these tissues has differential effects on neuropeptide expression by sympathetic neurons [76]. Fractionation of conditioned medium from heart cells has revealed the existence of several distinct factors which differentially affect peptide expression in cultured sympathetic neurons [77].

The studies of the neurotransmitter properties of sympathetic neurons developing in cell culture yielded two unexpected findings. First, postnatal, postmitotic neurons could change their neurotransmitter functions in not only a quantitative but also a qualitative manner; noradrenergic neurons could become cholinergic and neuropeptide expression could be altered. Second, the environment played a significant role in determining a functionally important aspect of the final phenotype of neurons, their choice of neurotransmitter(s). To assess whether the plasticity and environmental instruction revealed in the cell culture studies are part of the normal developmental repertoire of neurons, we have examined the development of cholinergic sympathetic neurons using the innervation of sweat glands as a model system.

Environmental regulation of transmitter status in vivo

Although the majority of principal neurons in sympathetic ganglia are noradrenergic, a small proportion are cholinergic. The best characterized are those that innervate sweat glands concentrated in footpads of laboratory animals. Several lines of evidence indicate that the sweat gland innervation is cholinergic and sympathetic in the rat: sweating evoked by nerve stimulation is blocked by local injections of atropine, a muscarinic antagonist [78]; footpad tissue contains high levels of choline acetyltransferase activity [79]; and labeled neurons are present in sympathetic ganglia following injection of fluorescent tracers in front or hind footpads. The innervation also contains immunoreactivity for two neuropeptides, vasoactive intestinal peptide (VIP) and calcitonin gene-related peptide (CGRP; [80]).

The neurotransmitter properties expressed by the developing sweat gland innervation, however, are strikingly different from those expressed by the mature innervation. When axons contact the developing glands during early postnatal development, they exhibit catecholaminergic markers [80, 81]. In

contrast, cholinergic and peptidergic markers are absent; choline acetyltransferase activity, acetylcholinesterase, and both VIP and CGRP immunoreactivities are undetectable [79, 80]. The neurotransmitter properties that characterize the mature innervation appear during the second and third postnatal weeks. As the gland innervation acquires cholinergic and peptidergic markers, detectable stores of catecholamines disappear, and immunoreactivity for the catecholamine synthetic enzymes, tyrosine hydroxylase and dopamine β-hydroxylase, decreases. It is clear that the changes in transmitter properties observed in the development of the sweat gland innervation take place in a single population of fibers rather than the loss of an early-arriving noradrenergic population and its replacement by a late-arriving population of cholinergic fibers [80, 81]. One of several lines of evidence comes from studies using 6-hydroxydopamine and guanethidine, adrenergic neurotoxins that are taken up selectively by catecholaminergic neurons and, when administered to neonatal rats, causes the destruction of peripheral noradrenergic cell bodies and nerve terminals. Following treatment of neonatal rats with either toxin, no acetylcholinesterase, VIP-IR, choline acetyltransferase activity or characteristic sympathetic varicosities are present in the glands, indicating that the mature cholinergic and peptidergic innervation is derived from the initial catecholaminergic innervation [82].

The neurotransmitter plasticity displayed by the developing sweat gland innervation is not unique. Examination of the development of neurotransmitter properties has disclosed a number of examples of altered expression of transmitter synthetic enzymes and neuropeptides, suggesting that qualitative changes in transmitter expression may be common. The most thoroughly studied example is the transient catecholaminergic cells of the gut [44, 45, 83, 84]. In the guts of embryonic, but not postnatal rats, cells are present which express tyrosine hydroxylase and catecholamine histofluorescence. These cells appear not to die but rather to acquire a different transmitter phenotype. It has proven difficult in this system, however, to establish what the final phentotype is.

Environmental cues play a role in specifying transmitter properties of sympathetic neurons *in vivo* as well as in culture. A logical source for such an instructional signal in the case of neurons that innervate sweat glands is their target. The possible role of the target, the sweat glands, in inducing the observed change in neurotransmitter-related properties has been tested directly in cross-innervation studies. In one series of experiments, sweat gland primordia in early postnatal rates were replaced with parotid gland, a target which receives noradrenergic sympathetic innervation. The innervation of the transplanted parotid gland retained intense catecholamine fluorescence and failed to develop choline acetyltransferase activity. Thus, the presence of the sweat gland appears to be required for the normal loss of catecholamines and induction of cholinergic function [85]. A second series of experiments took advantage of the topographical segregation of cholinergic and noradrenergic sympathetic targets in rat skin. Hairy skin normally receives noradrenergic sympathetic innervation, particularly of piloerectors and blood vessels [86], while the sweat gland-containing glabrous skin of the footpads normally receives cholinergic sympathetic innervation. By transplanting sweat gland-containing skin to the lateral thorax of neonatal rats, the sympathetic neurons which innervate noradrenergic targets in the hairy skin innervate sweat glands instead. The innervation of the transplanted sweat glands exhibits neurotransmitter-related properties appropriate for the novel target rather than normal hairy skin targets [87]. Although catecholamine-containing fibers have formed an intensely fluorescent plexus in the transplanted glands by 3 weeks, at 6 weeks only occasional faintly fluorescent fibers are present. Acetylcholinesterase, choline acetyltransferase activity and VIP are initially absent but appear in the sympathetic innervation of the transplanted glands between 3 and 6 weeks. It is noteworthy that the target influences the choice not only of the traditional transmitter but also of the neuropetide. Cross-innervation experiments involving sensory nerves suggest a similar target influence on peptide expression in this system as well [88]. These observations raise the possibility that at least some of the numerous instances of restriction of neuropeptide expression to a subpopulation of peripheral neurons that is correlated with innervation of a particular target [89–92] arise because of retrograde specification from the target.

The results of the transplantation experiments indicate that sweat glands induce neurons which innervate them to express cholinergic and peptidergic properties and suppress adrenergic properties. As a first step in determining the molecular nature of the transmitter-specifying signal(s) produced by the sweat glands, aqueous extracts have been made from developing and adult glands. Such extracts induce choline acetyltransferase activity in cultured sympathetic neurons, and experiments are underway to compare the properties of the activity in sweat gland extract with several of the factors identified as inducing cholinergic function in cultured sympathetic neurons including cholinergic differentiation factor, ciliary neurotrophic factor and MANS to determine whether it is one of these or a novel factor(s).

Regulation of neurotransmitter receptors and responsiveness

Cell–cell interactions which have been shown to regulate the expression of certain neurotransmitter properties, such as tyrosine hydroxylase (TH) [93, 94], in cultured chromaffin cells and neurons may also be important in regulating receptor concentration [95, 96]. Receptor regulation by hepatocytes provides an interesting example of this mechanism [97]. *In vivo*, β-adrenergic receptors (BAR) increase in concentration 5-fold between embryonic day (E) 16 and 20 and then decrease in concentration approximately 10-fold by the fourth postnatal week. During this period, there are no discernible changes in the pharmacological properties of the receptor and guanine nucleotides are found to be equally effective in modulating the affinity of the receptor in competition studies performed on homogenates of E20 and adult liver. The

expression of α_2-adrenergic receptors displays a different developmental time course; their concentration increases in a hyperbolic fashion approximately 5-fold between E16 and postnatal day 28 and then remains unchanged for up to 3 months. When liver cells are dissociated from adult rats and cultured at relatively low density, the concentration of BAR increases 4- to 8-fold. If, however, the hepatocytes are plated at 10-fold higher density, the concentration of receptors increases only 2-fold. Culturing the hepatocytes in serum-free medium, medium supplemented with 10% fetal bovine serum, or hormonally defined medium does not alter this pattern of receptor expression. Most interestingly, α-receptors disappear under all conditions tested. Thus, it appears that cell contact not only plays a role in determining the expression of adrenergic receptors in cultured hepatocytes but it also regulates adrenergic subtypes differentially.

The development of BAR in autonomic target tissues *in vivo* has been studied most extensively in the heart [98, 99] and parotid gland [100]. In most species examined, the development of cardiac BAR follows a similar pattern. They are first detectable early in development prior to the appearance of agonist-induced increases in intracellular cAMP or in heart rate. During the latter half of gestation and the early neonatal period, the BAR concentration increases sharply, exceeds the adult concentration by approximately 2-fold, and then subsequently decreases to adult levels. In the rabbit heart, after an initial period of nonresponsiveness, changes observed in receptor concentration are paralleled by changes in the maximal force of isoproterenol-stimulated myocardial contraction [99]. The timing of BAR expression is somewhat different in rat parotid gland [100]. β-Adrenergic receptors are first detectable shortly after birth and increase approximately 4-fold during the second and third weeks postnatal; the concentration of receptors acquired during postnatal development is maintained into adulthood. Like the heart, however, the parotid gland is initially refractory to adrenergic stimulation. Thus, in both tissues, receptors are expressed prior to functional responsiveness of the tissue. In the parotid gland, during the second and third postnatal week when responsiveness appears, there is a sharp increase in two cholera toxin specific substrates, a 43 and a 48 kD band. These findings suggest that the development of sensitivity is correlated more closely with the appearance of the G-protein than the receptor [101]. The presence of sympathetic innervation is not required for either receptor expression or responsiveness; animals treated with the adrenergic neurotoxin 6-hydroxydopamine from birth expressed a 50–60% greater concentration of β-adrenergic receptors and were approximately 1.7 times as sensitive to isoproterenol [101].

Like BAR, muscarinic cholinergic receptors appear early in the development of the chick heart, prior to the onset of physiological responsiveness [102–104]. Muscarinic ligand binding sites are first detectable by day 2.5 *in ovo* and their concentration, approximately 200 fmol/mg protein, does not change significantly between day 3 and 13 *in ovo* [102, 103]. During this period, however, the ability of the cholinergic agonist, carbachol, to attenuate an isoproterenol-stimulated elevation in cAMP increases about 4-fold [103]. The carbachol-induced attenuation is not due to alterations in the adrenergic response nor in adenyl cyclase activity since the ability of isoproterenol to stimulate cAMP did not change. Rather, the development of muscarinic responsiveness is correlated with the appearance of a pertussis toxin sensitive G-protein. Two pertussis toxin substrates, one of 39 kD and one of 41–42 kD, are present in homogenates of late embryonic chick heart [103, 104]. When G-protein was assayed by examining the inhibition of forskolin-activated adenyl cyclase by guanine nucleotides, the concentration of GMP-PNP, a nonhydrolyzable GTP analogue, required to produce a half-maximal inhibition of adenyl cyclase decreased from over 200 μM on day 4 to 18 μM on day 8 while the extent of inhibition remained constant [103]. This suggests that the functional properties of the G-protein have changed in parallel with the increase in muscarinic sensitive inhibition of adenyl cyclase. Direct examination of G-protein expression using pertussis toxin labeling, and immunoblotting indicate a significant increase in both forms, as well as a change in proportion of 39:42 from 0.45 to 1.25 over the same time period. Further analysis using two-dimensional gel electrophoresis demonstrated that there is a significant increase in the acidic form of the 39 kD α-subunit between day 4 and day 8 [104]. Thus, these studies suggest that sensitivity to cholinergic agonists in developing chick heart is regulated not by alterations in the density of ligand binding sites but rather by the appearance of G-proteins.

The lack of correlation between the presence of ligand binding sites and responsiveness is also apparent when distribution of muscarinic ligand-binding sites on atrial and ventricular myocardial cells is examined. In cultured chick heart myocytes the concentration of ligand-binding sites is almost identical in the two regions but only a small minority of ventricular cells respond to muscarinic agonists [105]. A similar discrepancy exists in the responsiveness of canine ventricle and atria to cholinergic agonist. Interestingly, denervation of canine ventricle for a period of 5–7 days leads to the development of sensitivity to acetylcholine [106]. Concurrent with this change the number of ventricular muscarinic receptors increases approximately 2-fold. Both the basal and oxotremorine stimulated GTPase activity increase [106]. Treatment of membranes prepared from control and denervated ventricle with pertussis toxin demonstrated a 2-fold increase in the amount of 39 and 41 kD substrates [106]. Immunoblotting confirmed these results, and in addition revealed that the beta subunit of the G-protein complex is increased by a similar factor. These studies suggest that the aberrant appearance of sensitivity as well as the onset of sensitivity to neurotransmitters during normal development are due to the induction of one or more G-proteins. Further experimentation is necessary to determine if this is indeed the case.

Although the factors that regulate cholinergic responsiveness in chick heart *in vivo* are not known,

evidence from cell culture studies raises the possibility that soluble factors play a role. For example, addition of saline extracts of chick brain to cultured ventricular cells leads to a significant increase in the number of ventricular cells responding to carbachol while the magnitude of the response and the resting potential remain unchanged as does the concentration of ligand binding sites determined autoradiographically [105]. In addition, certain lots of horse serum produce a significant increase in potassium efflux in cultured heart cells in response to muscarinic agonists; there is a parallel increase in the ability of guanine nucleotide to modulate the formation of the high-affinity complex [107]. In both cases, it is tempting to speculate that a G-protein(s) has been induced that is competent to couple the muscarinic receptor to the appropriate intracellular effector. This has not been shown directly, however, nor have the activities in either the chick brain extract or horse serum that affect the muscarinic cholinergic signal transduction system been characterized further. Interestingly, extracts of chick brain prepared in a similar manner produce a 2- to 3-fold increase in the concentration of nicotinic acetylcholine receptors on cultured myotubes [108]. A 42 kD glycoprotein, ARIA or acetylchloline receptor-inducing activity, has been purified from the extract that increases the rate of incorporation of acetylcholine receptors in chick myotube membranes and causes a 2- to 16-fold induction in the mRNA levels encoding the α-subunit of the nicotinic acetylcholine receptor [109–111].

In one autonomic target tissue, sweat glands, there is strong circumstantial evidence that acetylcholine itself triggers the development of secretory responsiveness. As summarized above, the sweat gland innervation is initially noradrenergic, and cholinergic function appears later. Pharmacological studies with cholinergic and adrenergic agonists and antagonists indicate that even in developing animals whose sweat gland innervation contains catecholamines, nerve-evoked sweat secretion is mediated by acetylcholine [78]. The onset of nerve- and agonist-induced sweating lags behind the development of cholinergic properties in the sweat gland innervation; glands which do not respond to nerve stimulation in developing rats are also unresponsive to muscarinic agonists. These observations raise the possibility that the cholinergic responsiveness of the gland cells is induced by the release of acetylcholine from the gland innervation, and experiments are in progress to test this hypothesis directly. Consistent with this notion is the finding that the glands of adults animals sympathetically denervated at birth do not sweat in response to cholinergic agonists [78]. In addition, when the innervation of the sweat glands is delayed, the development of responsiveness is also delayed and tightly linked to the onset of secretion following nerve stimulation [112]. Finally, others have shown that when glands are acutely denervated by cutting the sciatic nerve, the glands become unresponsive to cholinergic agonists; thus, maintenance of secretory responsiveness is dependent upon cholinergic innervation [113–115].

We have examined the expression of muscarinic ligand binding sites in sweat glands as a first step in determining how the innervation regulates secretory responsiveness in the target [116]. Ligand binding and competition studies reveal that mature innervated glands possess typical M_2 "glandular" receptors; recent *in situ* hybridization experiments suggest that it is most likely the m3 molecular subtype [117]. There is not a correlation between the presence of muscarinic binding sites and the ability of the glands to secrete in response to cholinergic agonists. During development, muscarinic binding sites appear well before the onset of secretory responsiveness and they are expressed at close to normal levels on both uninnervated and acutely denervated glands which are nonresponsive. Thus, it seems likely that one or more steps in the coupling of ligand binding to sweat secretion are innervation dependent.

Although the weight of the evidence suggests that the important developmental event in the acquisition of responsiveness in sweat glands and in chick heart is the induction of G-protein(s), the possible role of receptor subtype switching has not yet been addressed in either system or in any other autonomic target tissue. Recent molecular biological studies have revealed the existence of five muscarinic receptor genes [18–23], and expression studies have demonstrated that specific molecular subtypes preferentially couple to specific intracellular effectors. For example, the m1, m3 and m5 subtypes preferentially interact with phospholipase C and thus activate the phosphoinositide (PI) pathway, whereas the m2 and m4 receptors preferentially couple to the inhibition of adenyl cyclase [25, 26]. Based on such studies one would predict that specific pathways could be activated or inhibited selectively in target tissues. An example of this phenomenon has been described in rat brain by Gil and Wolfe [118]. In homogenates and slices of rat brain, acetylcholine inhibits the formation of cAMP and stimulates the PI pathway. These two effects display different K_i values for pirenzepine: 21 nM for phosphoinositide breakdown and 310 nM for the inhibition of adenyl cyclase. In traditional binding assays performed on rat brain, pirenzepine recognizes two sites with K_d values of 12 and 168 nM respectively. The nonselective antagonist atropine inhibits both the binding of pirenzepine and the two biochemical responses with equal affinity (approximately 1 nM). These results, taken together, suggest that the site which displays high affinity for pirenzepine is coupled to the PI pathway, while the site which exhibits a lower affinity for pirenzepine is coupled to the inhibition of adenyl cyclase. These studies raise the possibility that the responsiveness of a target tissue could be regulated not only by the presence or absence of the appropriate G-protein but also by the expression of a specific molecular receptor subtype. Thus, although muscarinic ligand binding sites are present in sweat glands and heart, they could represent a molecular subtype that is not coupled to the appropriate effector system. An analogy for such a switch has been described in the development of the ligand-gated nicotinic receptor at the neuromuscular junction. The gamma subunit replaces the epsilon in the multisubunit complex and causes changes in the biochemical, electrophysiological, and molecular properties of the receptor [119–121]. The existence

of immunological and molecular probes for receptor subtypes and G-proteins should provide evidence for or against this possibility in the near future.

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