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THE GLYCINE SYNAPTIC RECEPTOR IN THE MAMMALIAN CENTRAL NERVOUS SYSTEM

S.H. SNYDER (work done in collaboration with ANNE B. YOUNG)

Departments of Pharmacology and Experimental Therapeutics, and Psychiatry and the Behavioral Sciences, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, U.S.A.

Sir John Gaddum had a wide-ranging interest in all neurotransmitter candidates known in his time and made important contributions with respect to most of them. Even with his broad vision, Sir John might well have been amazed at the proliferation of new and unexpected transmitter candidates in the central nervous system. Through indirect pharmacological approaches, Gaddum attained considerable insight into receptor mechanisms for drugs and neurotransmitter. Today I will focus on one 'new' central nervous transmitter, glycine, and upon direct biochemical studies of its post-synaptic receptor.

In the mammalian central nervous system the most recognized neurotransmitters are acetylcholine, the catecholamines, noradrenaline and dopamine, and the indole amine, 5-hydroxytryptamine. Intensive studies of the synthesis, release and synaptic inactivation of the catecholamines have helped elucidate the mechanism of action of important drugs which affect behaviour, such as antidepressants, amphetamines, and phenothiazine tranquilizers (Carlsson, Kehr, Lindquist, Magnusson & Atack 1972; Schildkraut, 1973; Snyder, Banerjee, Yamamura & Greenberg, 1974). Similar investigation of the disposition of 5-hydroxytryptamine in the mammalian brain has helped clarify how psychedelic drugs act (Aghajanian, 1972). Yet despite the great amount of interest and clinical relevance of the biogenic amines, quantitatively they are not the major neurotransmitters in the brain and probably account for transmission at only a small percentage of central nervous synapses. Thus it is estimated that noradrenaline is a transmitter of only about 1% of synapses throughout the brain and, even in the hypothalamus, which contains the brain's highest level of noradrenaline only 5% of the nerve terminals contain noradrenaline (Fuxe, Hökfelt, Ritzen & Ungerstedt, 1968; Hökfelt, Jonsson & Lidbrink, 1970).

Dopamine levels in the brain are about the same as those of noradrenaline and in the corpus striatum, where dopamine is most enriched, only about 15% of the nerve terminals contain catecholamines (Hökfelt *et al.*, 1970). Autoradiographic studies suggest that even a smaller percentage of neurones in the brain contain 5-hydroxytryptamine (Kuhar & Aghajanian, 1973). Various workers estimate that acetylcholine nerve terminals involve only about 10% of brain synapses (McLennan, 1970).

In quantitative terms, amino acids now appear to be the major neurotransmitters in the mammalian central nervous system. Neurophysiologically, two classes of amino acids are recognized: excitatory amino acids (glutamic and aspartic acids, and cysteic and homocysteic acids) which depolarize most neurones in the mammalian central nervous system, and inhibitory amino acids (γ-aminobutyric acid (GABA), glycine, β-alanine and taurine) which hyperpolarize the neuronal cell membrane (Curtis & Watkins, 1960; Curtis, Hosli & Johnston, 1968a; Curtis, Hosli, Johnston & Johnston, 1968). Of this latter group, GABA and glycine are major candidates for the chemical mediation of inhibitory neurotransmission in the central nervous system. GABA is active throughout the neuroaxis whereas glycine affects neurones only in the spinal cord, brain stem and diencephalon but not in the cerebral cortex.

When brain slices or homogenates are incubated with low concentrations of radioactive GABA or glycine, nerve terminals which presumably utilize either amino acid as their neurotransmitter are selectively labelled by high affinity, sodium-dependent transport systems (Iversen & Neal, 1968; Roberts & Kuriyama, 1968; Iversen & Johnston, 1971; Logan & Snyder, 1972; Bennett, Logan & Snyder, 1972; 1973). Autoradiographic studies of such preparations reveal that the number of GABA accumulating neurones in the brain varies with different regions from about 27% in the cerebral cortex to 45% in the hippocampus, a considerably greater portion of synapses than contain either acetylcholine or the biogenic amines (Fuxe *et al.*, 1968; Hökfelt *et al.*, 1970; Iversen & Bloom, 1972; Iversen & Schon, 1973; Kuhar & Aghajanian, 1973). In the spinal cord, GABA and glycine appear to act at an equal number of synapses. Thus about 25% of spinal cord nerve terminals can be labelled with [^{3}H]-GABA and

25% can be labelled with [^{3}H]-glycine. When slices are exposed to both compounds, 50% of the nerve terminals are labelled (Iversen & Bloom, 1972).

With such an apparently large proportion of inhibitory synapses present, one might expect a comparable number of excitatory connections. What are the major excitatory neurotransmitters? The best candidates are the amino acids, glutamic and aspartic acids (Krnjević & Phillis, 1963; Werman, 1972) and possibly peptides, such as Substance P (Konishi & Otsuka, 1974; Phillis & Limacher, 1974). However, the evidence for specific compounds as excitatory neurotransmitters is less strong than for the inhibitory transmitters.

What criteria should be met for the identification of a neurotransmitter? Universally accepted ones are: (1) the compound is present in the appropriate neurones, (2) it is released when neurones are activated and perhaps most importantly, (3) its synaptic actions closely mimic those of the natural transmitter (Krnjević & Phillis, 1963; Werman, 1966; Werman, 1972). For amino acids, there is little doubt that all are present in neurones in the brain. To study an amino acid neurotransmitter biochemically, however, one must discriminate the 'transmitter pool' from possibly larger pools required for intermediary metabolism and protein synthesis. In the case of glycine, it appears possible to label selectively the neurotransmitter pool by means of the high affinity, sodium-dependent uptake system into 'glycinergic' nerve terminals (Iversen & Neal, 1968; Roberts & Kuriyama, 1968; Iversen & Johnston, 1971; Logan & Snyder, 1972, Bennett *et al.*, 1972; 1973). Nerve terminals which accumulate radiolabelled glycine by the high affinity uptake system can be separated physically from the majority of other nerve terminals by sedimentation of brain homogenates on linear sucrose gradients (Arregui, Logan, Bennett & Snyder, 1972). Glycine accumulated in this way can be released fairly selectively by electrical or potassium-induced depolarization (Bradford, 1970; Hopkin & Neal, 1971; Hamerstad, Murray & Cutler, 1971; Roberts & Mitchell, 1972; Mulder & Snyder, 1974).

In the central nervous system, the uptake and release of neurotransmitters must be analysed in whole tissue preparations rather than at individual synapses, thus making it difficult to fulfil rigorously the first two criteria. In contrast, methods for making intracellular recordings from spinal cord motor neurones have enabled investigators to compare directly the inhibitory synaptic actions of glycine with those of the natural inhibitory transmitter (Werman, Davidoff & Aprison, 1968; ten Bruggencate & Engberg, 1968; Curtis *et al.*, 1968a & b). Both glycine and GABA hyperpolarize spinal cord motor neurones with a reversal potential similar to that of the natural transmitter substance. Glycine and GABA can be discriminated pharmacologically. The inhibitory actions of glycine are antagonized by low concentrations of strychnine, while those of GABA are strychnine-resistant. Further, since strychnine blocks selectively the natural mediator for reciprocal or direct inhibition of spinal motorneurones, it appears probable that glycine is the natural inhibitory transmitter in these pathways (Owen & Sherrington, 1911; Bradley, Easton & Eccles, 1953; Larson, 1969; Curtis, Duggan & Johnston, 1971). Strychnine-sensitive glycine inhibition occurs in the brainstem as well as in the spinal cord and then decreases as one ascends the neuroaxis (Krnjević, Randić & Straughan, 1966; Biscoe & Curtis, 1967; Kelly & Krnjević, 1969; Larson, 1969; Curtis *et al.*, 1971; Tebecis & DiMaria, 1972). Interestingly, this regional distribution coincides with the regional distribution of endogenous glycine and of the high affinity neuronal uptake system for glycine (Table 1) (Iversen & Neal, 1968; Roberts & Kuriyama, 1968; Aprison, Shank & Davidoff, 1969a; Aprison, Shank, Davidoff & Werman, 1969b; Iversen & Johnston, 1971; Logan & Snyder, 1972; Bennett *et al.*, 1972; 1973).

The consensus is that glycine is the transmitter of certain small, inhibitory neurones in the brainstem and spinal cord and perhaps of some neurones in higher centres. Within the spinal cord, glycine hyperpolarizes motor neurones and decreases their frequency of firing (Werman *et al.*, 1968; ten Bruggencate & Engberg, 1968; Curtis *et al.*, 1968a & b). These inhibitory interneurones are apparently involved in the coordination of antagonistic muscle groups (Owen & Sherrington, 1911; Bradley *et al.*, 1953; Larson, 1969; Curtis *et al.*, 1971; Eccles, 1972). Thus when a monosynaptic excitatory reflex to extensor motor neurones is stimulated, the activity of the antagonistic flexor motorneurones must be suppressed. In this case, collaterals from the primary afferent inputs to the extensor motorneurones excite inhibitory interneurones (glycine interneurones) which in turn synapse on and hyperpolarize flexor motorneurones, thus inhibiting contraction of the flexor muscles. Strychnine, as an antagonist of glycine, abolishes this reciprocal inhibition which thereby allows a predominance of excitatory activity, causing tetany and convulsions.

Receptor binding

Until recently most biochemical studies of

neurotransmitters in the central nervous system have focussed on presynaptic events. Elegant techniques have been applied to studies of the synthesis of the biogenic amine transmitters, to measurements of their turnover rate, their depolarization-induced release from brain tissue and to their synaptic inactivation by reuptake into the nerve endings which have released them. Many of these approaches have proved valuable in studying amino acid putative neurotransmitters including GABA, glutamic and aspartic acids and glycine. However, little is known of the biochemical properties of the postsynaptic receptors for central nervous transmitters. In the electric organ of the electric eel *Torpedo marmorata* and at vertebrate neuromuscular junctions, a variety of ligands have been employed to demonstrate binding to the nicotinic cholinergic receptor (O'Brien, Eldefrawi & Eldefrawi, 1972; Hall, 1972; Potter & Molinoff, 1972; DeRobertis & Schacht, 1974), and in mammalian brain, it is now possible to identify biochemically muscarinic cholinergic receptors (Soudijn, van Wijngaarden & Ariens, 1973; Burgen, Hiley & Young, 1974; Yamamura, Kuhar, Greenberg & Snyder, 1974; Yamamura & Snyder, 1974).

Using radioactive strychnine, we have successfully measured glycine receptors in the mammalian central nervous system (Young & Snyder, 1973; Snyder, Young, Bennett & Mulder, 1973; Young & Snyder, 1974a,b). Strychnine was chosen as a ligand rather than glycine to avoid possible binding of glycine to presynaptic membrane fragments associated with the glycine uptake system and because neurophysiological studies had suggested that strychnine had a much greater affinity for the glycine receptor than glycine itself. Using a rapid centrifugation technique and tritiated strychnine of high specific activity, we can demonstrate strychnine binding to synaptic membrane preparations of spinal cord and brain stem of the rat and monkey (Young & Snyder, 1973; Snyder *et al.*, 1973; Young & Snyder, 1974a,b). Strychnine binding is most enriched in the synaptic membrane fraction, which contains both pre- and postsynaptic membrane fragments, supporting the notion that strychnine binding is associated with a synaptic receptor site.

In addition, bound [^{3}H]-strychnine is displaced by glycine. Half maximal displacement occurs at about 25 μM glycine with maximal displacement at 1.0 mM glycine. As a measure of strychnine binding to the glycine receptor we define 'specific [^{3}H]-strychnine binding' as the amount of bound [^{3}H]-strychnine which can be displaced by 1 mM glycine. Binding not displaceable by glycine, we define as 'nonspecific strychnine binding'. The ratio of specific to nonspecific binding is about 5-6 at 1-4 nM [^{3}H]-strychnine concentration. The specific binding of [^{3}H]-strychnine is a saturable process with half maximal binding at about 2.7 nM, four orders of magnitude lower than the comparable value for glycine. Nonspecific binding is not saturable and increases linearly with increasing amounts of strychnine. Double-reciprocal and Scatchard analyses indicate that strychnine binds to a single population of receptor sites. The number of strychnine binding sites in the rat spinal cord is about 39 pmol/gram. Interestingly, this is about the same as the number of muscarinic cholinergic receptors in rat brain (Soudijn *et al.*, 1973; Burgen *et al.*, 1974; Yamamura, *et al.*, 1974; Yamamura & Snyder, 1974).

The fact that strychnine binding saturates at low concentrations and can be displaced by glycine is not sufficient evidence to ensure that binding occurs selectively to postsynaptic glycine receptors. As another criterion for specificity, we examined the regional distribution of strychnine binding (Table 1) (Young & Snyder, 1973; Snyder *et al.*, 1973; Young & Snyder, 1974a). As already mentioned, endogenous levels of glycine are highest in the spinal cord and brainstem, somewhat lower in the midbrain and diencephalon and lowest in the cerebellum and cerebral hemispheres. Glycine's ability to mimic natural inhibitory synaptic actions is greatest in the brainstem and spinal cord and less in higher centres (Krnjević *et al.*, 1966; Biscoe & Curtis, 1967; Kelly & Krnjević, 1969; Tebécis & DiMaria, 1972). Thus, one might expect glycine receptors to be most abundant in the spinal cord and brainstem and less in higher centres such as the cerebral cortex. Glycine receptor binding is indeed greatest in the spinal cord and almost as high in the medulla oblongata-pons (Table 1). Receptor binding in the midbrain is only about half that in the medulla oblongata-pons and thalamus, and negligible binding can be demonstrated in the cerebellum, hippocampus, corpus striatum and cerebral cortex. This distribution closely parallels that of endogenous glycine and high affinity glycine uptake systems. Within the spinal cord, endogenous glycine is highest in the cervical and lumbar regions, areas which contain a large proportion of inhibitory interneurones for the regulation of the innervation to the upper and lower extremities. Accordingly, in the monkey's spinal cord specific [^{3}H]-strychnine binding is highest in the cervical and lumbar grey matter and somewhat less in the thoracic grey matter. Receptor binding is undetectable in the white matter of the spinal cord, where few if any synapses occur.

To confirm further the specificity of [^{3}H]-

Table 1 Regional distribution of glycine and strychnine neurophysiological effects, endogenous glycine, glycine high affinity uptake systems and specific [^{3}H]-strychnine binding

	Strychnine-sensitive inhibition[1]	*Glycine hyperpolarization of neurones*[2]	*Endogenous glycine levels*[3] *(μmol/g)*	*High affinity glycine uptake*[4]	*Specific strychnine binding*[5] *(ct min^{-1} mg^{-1} protein)*	
					Rat	*Monkey*
Lumbar spinal cord	+++	+++	4-5	+		1042
Thoracic spinal cord	++	++	3-4	+	2315	775
Cervical spinal cord	+++	+++	4-5	+		886
Medulla oblongata-pons	++	++	3-4	+	1712	–
Midbrain	(?)	(?)	1-2	–	863	–
Thalamus and hypothalamus	(?)	(?)	1	–	500	–
Cerebellum	0	0	1	–	80	–
Cerebral cortex	(±)	(±)	1	–	80	–

1. Data from Curtis *et al.*, 1971. 2. Data from Werman *et al.*, 1968. 3. Data from Aprison *et al.*, 1969a. 4. Data from Bennett *et al.*, 1973. 5. Data from Young & Snyder, 1973.

Table 2 Neurophysiological actions of amino acids and their effects on specific [^{3}H]-strychnine binding

Compound	*% Displacement of bound [^{3}H]-strychnine*** 10^{-5} M	10^{-4} M	10^{-3} M	*Neurophysiological activity of amino acids**	*Strychnine antagonized depression of spinal dorsal horn interneurones*
α-Amino acids					
Glycine	50	84	100	– – – –	yes
L-α-Alanine	5	40	64	– –	yes
L-Serine	6	17	56	–	yes
L-Cystathionine	–	–	–	(–)	yes
Aminomethane sulphonic acid	0	7	38	0	yes
Proline	0	12	61	not tested	not tested
Glutamic acid	0	0	0	++++	no
Aspartic acid	0	0	0	++++	no
Tryptophan	–	–	47	not tested	not tested
Phenylalanine	–	–	31	not tested	not tested
β-Amino acids					
β-Alanine	50	85	100	– – –	yes
DL-β-Aminoiso butyric acid	26	47	100	– –	yes
Taurine	14	68	100	– –	yes
γ-Amino acids					
γ-Aminobutyric acid	0	9	24	– – –	no
γ-Amino-β-hydroxybutyric acid	–	–	21	– –	no
Higher -amino acids					
Δ-Aminovaleric acid	–	–	28	– –	no
ε-Aminocaproic acid	–	–	20	–	no

Amino acids having no effect at 10^{-3} M: isoleucine, cysteine, valine, histidine, methionine, threonine, lysine, arginine, and leucine.

* Curtis *et al.*, 1961; 1968: (–) indicates relative depressant activity relative to glycine. (+) indicates relative excitatory activity.

** See Young & Snyder, 1973, 1974a.

strychnine binding we compared the ability of a variety of amino acids to displace bound [^{3}H]-strychnine with their ability to mimic the strychnine-sensitive neurophysiological actions of glycine (Table 2). β-Alanine and glycine are the most potent displacers of [^{3}H]-strychnine and are also the most potent amino acids neurophysiologically. Taurine, DL-β-aminoisobutyric acid and L-α-alanine are second most potent both neurophysiologically and as inhibitors of [^{3}H]-strychnine binding. Serine is somewhat less effective as a displacer of bound strychnine and has a limited ability to mimic glycine neurophysiologically. Most of the other amino acids examined have negligible ability to block strychnine binding and are unable to reproduce the synaptic actions of glycine in neurophysiological studies.

The close parallel between the regional distribution of specific strychnine binding, endogenous glycine levels and glycine's neurophysiological actions, as well as the impressive correlation between the neurophysiological actions of glycine-like amino acids and their ability to inhibit [^{3}H]-strychnine binding, indicate that specific [^{3}H]-strychnine binding involves synaptic recpetor sites for glycine.

Apparent interactions of strychnine with the ionic conductance mechanism of the glycine receptor

It is thought that neurotransmitters interact with a specific recognition site on the postsynaptic receptor thereby triggering alterations in a closely associated ion channel or 'ionic gate'. This causes a change in the permeability of the membrane to a particular ion or ions, and an alteration in membrane conductance which results in either neuronal depolarization or hyperpolarization. For excitatory neurotransmitters, such as acetylcholine at the neuromuscular junction, the ion mediating the depolarizing effects of acetylcholine seems to be predominantly sodium (Fatt, 1954; Katz, 1966). For inhibitory neurotransmitters such as GABA and glycine the transmitter appears to bring about an increase in conductance to chloride and possibly potassium which effects a hyperpolarization of the membrane (Ito, Kostyk & Oshima, 1962; Eccles, 1964). A major aim of biochemical studies of neurotransmitter receptors involves the identification of the receptor recognition site and ionic conductance mechanisms in order to ascertain on a molecular basis how neurotransmitter recognition at one site triggers an alteration in the ionic permeability of the neuronal membrane. A variety of ligands can be used to label the recognition site of neurotransmitter receptors (O'Brien *et al.*, 1972; Hall, 1972; Potter & Molinoff, 1972; Soudijn *et al.*, 1973; Burgen *et al.*, 1974; DeRobertis & Schacht, 1974; Yamamura *et al.*, 1974; Yamamura & Snyder, 1974) and recently, the neurotoxin, histrionicotoxin, has been shown to interact with the ionic conductance modulator of the nicotinic acetylcholine receptor at neuromuscular junctions (Albuquerque, Barnard, Chiu, Lapa, Dolly, Jansson, Daly & Witkop, 1973). However, it is not yet possible to study biochemically the binding of histrionicotoxin to these sites.

We first suspected that glycine and strychnine might bind to different sites on the glycine receptor when we examined the influence of a variety of protein modifying reagents on [^{3}H]-strychnine binding and its displacement by glycine (Table 3). Diazonium tetrazole and acetic anhydride have very little effect on the total amount of [^{3}H]-strychnine bound to synaptic membranes. However, both of these reagents markedly interfere with the ability of glycine to displace [^{3}H]-strychnine binding. By contrast, tetranitromethane and dinitrofluorobenzene inhibit the total binding of [^{3}H]-strychnine but glycine and strychnine still displace the remaining bound [^{3}H]-strychnine to similar extents. The differential influence of diazonium tetrazole and acetic anhydride on strychnine and glycine interactions with the receptor suggest that glycine might displace strychnine by acting at site other than the strychnine binding site.

If the effect of diazonium tetrazole is to modify residues that are necessary for the interaction of the glycine site with a distinct strychnine binding site, it might be possible to 'protect' these residues by pretreating the membranes with diazonium tetrazole in the presence of glycine. Indeed, glycine can completely protect the membranes from the effects of diazonium tetrazole (Table 3). By contrast strychnine, even at 1 mM concentration, affords no protection of the membranes from diazonium tetrazole treatment. In fact, it seems to enhance slightly the ability of the reagent to inhibit glycine displacement.

These data suggest that glycine and strychnine bind to two separate but mutually interacting sites. If this is so, analysis of their binding interactions at various concentrations might be anticipated to demonstrate cooperative interactions. Hill plots for the displacement of [^{3}H]-strychnine or glycine are linear with an n for displacement with strychnine of 1.0, while the n for displacement with glycine is 1.7 (Young & Snyder, 1974b). This suggests that glycine interacts with strychnine in a cooperative fashion.

If the preferential influence of diazonium tetrazole on glycine's displacement of strychnine binding derives from an interference with inter-

actions between glycine and strychnine binding sites, one might expect diazonium tetrazole pretreatment to alter the cooperative effects of glycine on strychnine binding. Acordingly, Hill plots were performed for the displacement of [^{3}H]-strychnine binding by glycine using synaptic membranes pretreated with low concentrations of diazonium tetrazole which reduce specific strychnine binding less than 30%. Interestingly, diazonium tetrazole pretreatment of the synaptic membranes reduces the Hill coefficient for strychnine displacement by glycine from a value of 1.7 in control membranes to values of 1.2 and 1.1 after pretreatment with 0.25 mM and 0.75 mM diazonium tetrazole respectively. Acetic anhydride pretreatment similarly reduces the Hill coefficient for displacement of strychnine binding by glycine from 1.7 to 1.2 (Young & Snyder, 1974a).

Finally, we measured the dissociation rate of the [^{3}H]-strychnine-receptor complex before and after pretreatment with two concentrations of diazonium tetrazole. The dissociation of [^{3}H]-strychnine is unaffected by diazonium tetrazole pretreatment when measured after the addition of unlabelled strychnine at time zero. However, the dissociation rate when displacement is measured after the addition of glycine is considerably retarded in the diazonium tetrazole-pretreated membranes. The half-life for dissociation in control membranes after glycine addition is 45 s, while the half-lives using membrane preparations pretreated with 0.25 mM and 0.75 mM diazonium tetrazole are 56 s and 66 s, respectively (Young & Snyder, 1974a). These results indicate further that glycine and strychnine binding can be differentiated and that diazonium tetrazole may interfere with interactions between their binding sites.

Thus strychnine and glycine apparently bind to different portions of the receptor. Glycine presumably interacts with the glycine recognition site. To what structure does strychnine bind? Our first clue came in evaluating the effects of a variety of ions on [^{3}H]-strychnine binding. We found that in the absence of sodium, physiological concentrations of chloride markedly reduced specific [^{3}H]-strychnine binding. Since glycine-induced hyperpolarization of the neuronal membrane results primarily from a change in conductance of the membrane to chloride, we wondered whether strychnine might bind to the chloride ionic conductance modulator of the glycine receptor. Accordingly, we studied the influences of a variety of anions on strychnine binding. Numerous ions have been evaluated for their ability to substitute for chloride neurophysiologically during transmitter-induced hyperpolarization of the neuronal membrane. Thus when certain anions are injected iontophoretically into spinal motor neurones, the cell is hyperpolarized beyond the reversal potential for the transmitter-induced conductance change in the neuronal membrane. When inhibitory inputs to the cell are then stimulated, a depolarization results, presumably because the ion can traverse the chloride ionic gate which is 'opened' by neurotransmitter-receptor interactions. Ions which cannot traverse these channels fail to cause a reversal of the inhibitory postsynaptic potential. Neurophysiologically, ions behave in an all or none fashion; either they are fully effective in reversing the inhibitory postsynaptic potential or they are

Table 3 Effects of protein modifying reagents on specific [^{3}H]-strychnine binding

Reagent		*Total [^{3}H]-strychnine binding (% control)*	*Strychnine displaceable [^{3}H]-strychnine binding (% control)*	*Glycine displaceable [^{3}H]-strychnine binding (% control)*
None		100	100	100
2,4-Dinitrofluorobenzene	4.0 mM	37	28	26
Tetranitromethane	5.0 mM	52	42	40
N-ethylmaleimide	4.0 mM	93	92	91
Acetic anhydride	10 mM	100	98	69
	25 mM	98	84	29
	50 mM	70	51	10
Diazonium tetrazole	0.25 mM	100	98	90
	0.75 mM	95	93	70
	1.00 mM	90	85	33
	1.25 mM	90	60	18
Diazonium tetrazole,	1.0 mM			
+ glycine	1.0 mM	96	94	81
+ strychnine	1.0 mM	80	64	0

Data are modified from Young & Snyder, 1974a.

ineffective. The neurophysiological action of the anions appears to correlate with their hydration radii such that neurophysiologically active anions have hydration sizes similar to or smaller than chloride, while anions with larger hydration radii are inactive. The only striking exception to this sequence is formate, whose hydration radius is 40% larger than that of chloride, but which is effective in inverting inhibitory postsynaptic potentials, while several other anions with hydration radii larger than chloride but smaller than formate are neurophysiologically inactive (Ito *et al.*, 1962; Eccles, 1964).

We evaluated a series of 14 anions whose neurophysiological actions on inhibitory postsynaptic potentials in spinal motorneurones are known (Young & Snyder, 1974b) (Table 4). Their ability to inhibit [^{3}H]-strychnine binding to spinal cord synaptic membranes correlates closely with their neurophysiological capacity to invert inhibitory postsynaptic potentials in the mammalian spinal cord. Seven neurophysiologically active ions are able to inhibit specific [^{3}H]-strychnine binding, while seven neurophysiologically ineffective ions fail to alter strychnine binding. While effects on strychnine binding tend to correlate with the hydration radii of the ions, the correlation is better with the neurophysiological properties, since formate, whose hydration radius is 40% greater than chloride, is active neurophysiologically and in inhibiting strychnine binding.

The close correlation between the effects of anions on strychnine binding and on inhibitory postsynaptic potentials in mammalian spinal cord suggests that strychnine binding is closely associated with the chloride ionic conductance mechanism of the glycine receptor. Whether or not strychnine binds directly to the chloride 'gate' is unclear. Hill plots of the displacement of [^{3}H]-strychnine by chloride, iodide and bromide give coefficients of 2.3-2.7, suggesting that chloride interacts with strychnine binding in a cooperative fashion, which might mean that the two compounds bind to different sites. It is also possible, however, that strychnine binds to a site such as the exterior of the chloride channel and, to displace it, several chloride molecules must occupy the interior of the channel. In any case, we know of no feature of the glycine receptor other than the ionic gating mechanism which can account for the close relationship between anion effects on strychnine binding and on inhibitory postsynaptic potentials.

The fact that strychnine binding might be associated with the ionic conductance mechanism for chloride while glycine interacts with the glycine recognition site provides us with a tool to examine the interactions between these two portions of the glycine receptor. Recently, we have developed techniques for measuring the direct binding of glycine to the receptor and have also succeeded in solubilizing the glycine receptor. It may now be possible to separate physically the glycine and strychnine binding moieties of the receptor, to characterize them individually and to study their interactions.

Table 4 Effects of various anions on specific [^{3}H]-strychnine binding and reversal of the inhibitory postsynaptic potential (ipsp)

*Anion**	*ED_{50} for inhibition of specific [^{3}H]-strychnine binding*	*Hydration radii (22)*	*Reversal of the ipsp (22)*
Formate	160	1.35***	+
Bromide	235	0.94	+
Chloride	260	0.95	+
Iodide	300	0.96	+
Nitrate	335	1.03	+
Perchlorate	440	1.09	+
Thiocyanate	620	1.12	+
Fluoride	—**	1.33	—
Bicarbonate	—**	1.65	—
Acetate	—**	1.80	—
Sulphate	—**	1.85	—
Sulphite	—**	2.05	—
Phosphate, monobasic	—**	2.05	—
Phosphate, dibasic	—**	2.56	—

* Ammonium salts of all anions were used (Data are from Young & Snyder, 1974b).
** No inhibition detectable at 1.0 M concentrations.
*** Hydration radii expressed relative to potassium's which is given the value of unity (Eccles, 1964).

The notion that glycine interacts in a cooperative fashion with strychnine binding and that the cooperativity may be between the recognition site and the ionic gate of the receptor may have implications for elucidating synaptic features of other neurotransmitters. In several systems, the neurophysiological synaptic actions of glycine, acetylcholine and GABA display cooperative features (Changeux & Podleski, 1968; Takeuchi & Takeuchi, 1969; Davidoff, Aprison & Werman, 1969; Brookes & Werman, 1970; 1973). At the neuromuscular junction of the crayfish, Takeuchi & Takeuchi (1969) have examined interactions between GABA and picrotoxin, a GABA receptor blocker. GABA hyperpolarizes the crustacean neuromuscular junction in a cooperative fashion with Hill coefficients of 1.9, whereas the Hill coefficient for picrotoxin blockade of GABA effects is 1.0 and picrotoxin appears to block GABA effects non-competitively. This is very reminiscent of our data differentiating the glycine and strychnine binding sites of the glycine receptor. In further analogy, Takeuchi & Takeuchi (1969) observe that increasing concentrations of chloride reduce the ability of picrotoxin to block GABA effects. Half maximal inhibition occurs at about 200 mM chloride concentrations, similar to the half maximal concentration needed for inhibiting strychnine binding to the glycine receptor. Conceivably, at this GABA receptor the cooperative interactions take place between the GABA recognition sites and the chloride ionic gate, where picrotoxin may act. Such detailed neurophysiological studies have not yet been performed for glycine.

Cooperativity of neurotransmitter actions may serve a valuable physiological function. Because of the sigmoid character of the dose-response curve, the membrane potential of the postsynaptic neurone would be protected from small amounts of transmitter which might leak into the synaptic cleft but fail to trigger a change in membrane response because of the cooperativity of the dose-response curve. Similarly, the mechanism responsible for terminating the synaptic actions of a transmitter need not be 100% efficient, since it would only have to reduce the concentration of transmitter in the synaptic cleft below a threshold level and would not be obliged to 'sweep' the cleft free of every last transmitter molecule.

Further, the sigmoidicity of the transmitter-receptor dose-response curve may have implications for the mechanism of action of certain pharmacological agonists. Thus doses of drug that do not in themselves activate the receptor complex may have marked effects on function by increasing the ambient level of active agent in the synaptic cleft to near threshold levels. Then, when a natural pathway is stimulated, a smaller amount of transmitter release is necessary to reach threshold concentrations and activate the postsynaptic receptor. By such mechanisms, an agonist drug, in proper doses, might have no intrinsic effect on neuronal function but would instead serve to enhance the effectiveness of transmission in certain endogenous transmitter pathways.

Mechanism of benzodiazepine action

Many psychotropic drugs are known to act via the biogenic amine neurotransmitters. Except for convulsants such as picrotoxin and strychnine, no drugs have hitherto been shown to act as agonists or antagonists of amino acid neurotransmitters. The availability of a simple and specific assay for the glycine receptor prompted us to screen a large number of psychotropic drugs for the ability to inhibit [^{3}H]-strychnine binding (Young, Zukin & Snyder, 1974). Many drugs examined had negligible effects on strychnine binding (Table 5). The benzodiazepines, such as diazepam (Valium), are as potent in inhibiting [^{3}H]-strychnine binding as glycine itself (Young *et al.*, 1974). Diazepam inhibits strychnine binding 50% at a concentration of about 26 μM. However, the fact that a drug exerts an effect upon a particular biochemical parameter by no means establishes that this is the mechanism of the drug's therapeutic action. To determine whether benzodiazepine's interactions with the glycine receptor are related to the clinical effects of the drug, we examined a series of 21 benzodiazepines whose pharmacologic activities have been extensively evaluated in animal tests and in humans (Zbinden & Randall, 1967; Morselli, Cassano, Piacidi, Muscettola & Rizzo, 1973) (Table 5). There is a 50-fold variation in the potency of these drugs in inhibiting [^{3}H]-strychnine binding with values of half maximal inhibition ranging from 19 μM to more than 1000 μM. Diazepam (Valium) and chlordiazepoxide (Librium), the two most frequently prescribed drugs in the United States have ED_{50} values of 26 μM and 200 μM respectively.

To determine if the interactions of benzodiazepines with the glycine receptor relate to their clinical activities, we compared the clinical potency of the drugs in pharmacological tests that predict clinical activity with their potency in displacing [^{3}H]-strychnine binding (Table 5). The ability of the drugs to displace bound [^{3}H]-strychnine correlates very closely with their potencies in a 'human bioassay' based on the minimal dose at which 50% of the subjects experience subjective effects. Similar close correlations occur between strychnine displacement and the potencies of the

drugs in several behavioural tests in animals. These tests, such as the mouse antifighting, monkey taming, mouse and cat muscle relaxation, antipentylenetetrazole seizure and rat continuance avoidance tests are effective predictors of the drug potencies in humans. Considerably lower correlations are obtained with discrete trial conditioning, with the effects of the drugs on escape failure from electric shock, and convulsions elicited by minimal or maximal electroshock. However, these tests are also less effective predictors of benzodiazepine action in humans (Zbinden & Randall, 1967; Morselli *et al.*, 1973).

The close correlation between the pharmacological activity of the benzodiazepines and their displacement of strychnine binding strongly suggests that the drugs exert their pharmacological activities by interacting with the glycine receptor. However, it is still conceivable that the relative pharmacological potencies of the drugs *in vivo* relate simply to their ability to reach brain receptors because of factors such as lipid

Table 5 Benzodiazepines: correlation of [^{3}H]-strychnine binding displacement with behavioural effects

Behavioural test	*No. of drugs tested*	*Statistical significance* (P)	*Spearman correlation coefficient*
Human bioassay	20	<0.001	0.74
Fighting mouse test	21	<0.001	0.71
Antipentylene tetrazole test, mice	20	<0.004	0.63
Continuous avoidance, shock rate increase, rat	20	<0.004	0.63
Cat muscle relaxation	19	<0.005	0.67
Monkey taming	19	<0.005	0.65
Mouse muscle relaxation	20	<0.005	0.61
Antimaximal electroshock, mice	19	<0.05	0.46
Discrete trials 'trace' avoidance, noise response failure, rat	14	<0.10	0.51
Antiminimal electroshock, mice	19	<0.20	0.33
Continuous avoidance, escape failure, rat	19	<0.45	0.20

Central muscle relaxants without effect on [^{3}H]-strychnine binding at 10^{-2} M

Carisoprodol	Mephenesin
Chloroxazone	Methaqualone
Meprobamate	Tybamate
Methocarbamol	

Drugs without effect on [^{3}H]-strychnine binding at 10^{-4} M

Tetracycline	Ketamine
Aminosalicylic acid	Lithium carbonate
Nicotinamide	Ethanol
Pyridoxal HCl	Reserpine
Dilantin	Benactyzine
α-Naphthylthiourea	Haloperidol
Urea	Hydroxyzine
Acetazolamide	Diethyl carbamazine
Ethoxyzolamide	Lidocaine HCl
Methyl-DOPA	Dichlorphenamide
Ergocristine	Chlorphenesin
Methysergide	Ethosuximide
Hippuric acid	Phenacemide
Melatonin	Carbamazepine
Tolbutamide	Acetophenetidine
Alloxan	

Data are adapted from Young *et al.*, 1974.

solubility. Such effects might also determine the access of the drugs to strychnine binding sites in our synaptic membrane preparations. If this is the case, the same factors should govern interactions of benzodiazepines with other central nervous system receptors *in vitro*. To examine this possibility, we measured the ability of 10 of the benzodiazepines, including the most and least potent, to interact with opiate and muscarinic cholinergic brain receptors (Soudijn *et al.*, 1973; Pert & Snyder, 1973; Burgen *et al.*, 1974; Yamamura *et al.*, 1974; Yamamura & Snyder, 1974; Pasternak & Snyder, 1974). Displacement of [^{3}H]-dihydromorphine binding to opiate receptor sites or displacement of binding of [^{3}H]-quinuclidinyl benzilate to muscarinic cholinergic receptors in membrane preparations of rat brain requires concentrations of benzodiazepines 10-50 times higher than those needed for displacement of bound [^{3}H]-strychnine. Further, the inhibition of opiate and muscarinic receptor binding by benzodiazepines fails to correlate with any of the pharmacological tests. Thus, it is highly unlikely that the correlation of pharmacological activity of benzodiazepines with their ability to displace [^{3}H]-strychnine binding can be explained simply by nonspecific membrane interactions.

Drugs other than the benzodiazepines, most notably propanediols such as meprobamate (Miltown, Equanil), also exert muscle relaxant effects. These agents fail to displace [^{3}H]-strychnine binding in concentrations as high as 10 mM. However, the bearing of these data on the mechanism of action of these drugs must be viewed with caution, since these drugs are pharmacologically quite weak *in vivo* compared to the benzodiazepines. For example, in clinical usage meprobamate is less than 1% as potent as diazepam.

The major clinical actions of the benzodiazepine drugs are relief of anxiety and muscle relaxation. Since the anxiety relieving properties of these drugs are closely correlated with their muscle relaxing actions, the latter might be responsible for the amelioration of anxiety. Alternatively, antianxiety and muscle relaxant effects might be exerted at different parts of the central nervous system but utilize a common mechanism. Glycine neurones and receptors exist in both the spinal cord and brainstem, and in diencephalic structures. The concentrations of benzodiazepines in the blood and central nervous system at pharmacologically active doses (Zbinden & Randall, 1967; Morselli *et al.*, 1973) is similar to the concentration required to displace [^{3}H]-strychnine binding. Because of the impressive correlation of the pharmacological activity of the benzodiazepines and their interactions with central nervous glycine receptors, we propose that these drugs may produce their antianxiety, anticonvulsant and muscle relaxant effects by mimicking the actions of glycine at its receptor sites in the central nervous system. Conceivably, muscle relaxant actions involve an enhancement of glycine-mediated inhibition in the spinal cord, while antianxiety effects result from enhanced synaptic inhibition in the brain stem or higher centers. Influences of benzodiazepines on limbic system activity (Zbinden & Randall, 1967) might involve glycine receptors in limbic structures (Young & Snyder, 1973; Snyder *et al.*, 1973; Young *et al.*, 1974) or might arise secondarily from effects on brainstem receptors. The effects of benzodiazepines on seizure activity, both cortical and subcortically induced, may result from potentiating inhibitory pathways from the brainstem reticular activiting systems.

Concluding remarks

The ability to identify biochemically the synaptic receptor for glycine in the central nervous system has valuable implications for understanding synaptic mechanisms and for elucidating the mechanism of action of certain drugs. The notion that cooperative interactions exist between the glycine recognition site and its ionic conductance mechanism is reminiscent of neurophysiological results obtained for interactions between GABA and picrotoxin. Perhaps such synaptic mechanisms may exist for a variety of neurotransmitters. Similarly the apparent ability of the benzodiazepine drugs to exert their clinical effects by mimicking glycine at its receptors suggests that studying the influence of psychotropic drugs on synaptic actions of other amino acid neurotransmitters will be heuristic.

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