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Glycine stimulates striatal dopamine release in conscious rats

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1 Glycine is an inhibitory neurotransmitter in the spinal cord and brainstem. The mechanism of this inhibition is via binding of glycine to specific receptors, increasing transmembrane Cl^- conductance and hyperpolarizing neurones. Strychnine selectively antagonizes these effects. The role of glycinergic neurones in supraspinal regions is poorly understood.

2 Effects of glycine on release of catecholamines in the striatum were examined by microdialysis in freely-moving rats. Transcription of the genes encoding strychnine-sensitive glycine receptors was assessed in the striatum and substantia nigra, by use of reverse transcription followed by the polymerase chain reaction.

3 Glycine administered via the microdialysis probe dose-dependently increased concentrations of dopamine and its metabolites, dihydroxyphenylacetic acid and homovanillic acid, in the perfusate, indicating increased local release and metabolism of dopamine. Strychnine markedly attenuated these responses. Whereas striatal tissue did not contain mRNA for either the adult or neonatal form of strychnine-sensitive glycine receptor, nigral tissue contained a message for the adult form.

4 The results suggest that dopaminergic cells in the substantia nigra synthesize strychnine-sensitive glycine receptors and transport the receptors to terminals in the striatum. Occupation of the glycine receptors then exerts a net stimulatory effect on striatal dopamine release *in vivo*.

Keywords: Glycine; strychnine; microdialysis; substantia nigra; dopamine; dihydroxyphenylacetic acid (DOPAC); homovanillic acid (HVA); polymerase chain reaction

Introduction

Glycine has long been considered to be an inhibitory neurotransmitter in the central nervous system (Curtis & Malik 1968; Krnjevik, 1974). As with γ-aminobutyric acid (GABA), a prototypical inhibitory transmitter, glycine hyperpolarizes neurones by increasing membrane chloride conductance (Bormann, 1988). Strychnine blocks glycine- but not GABA-induced neuronal inhibition (Young & Snyder, 1973).

In rat striatal slices previously exposed to [^{3}H]-dopamine, both glycine and GABA increase spontaneous [^{3}H]-dopamine release (Giorguieff *et al.*, 1978; Kerwin & Pycock, 1979). Analogously, after loading brain slices from other regions with [^{3}H]-noradrenaline, [^{3}H]-acetylcholine or [^{3}H]-dopamine, addition of glycine to the medium increases release of the radioactivity, and strychnine blocks the glycine-evoked release. These stimulatory effects have been attributed to blockade of local inhibitory interneurones. Recent findings, however, have indicated direct stimulatory effects of glycine on release of endogenous catecholamines from isolated chromaffin cells (Yadid *et al.*, 1991; 1992). Moreover, radioligand binding (Yadid *et al.*, 1989) and autoradiographic studies (unpublished data) have confirmed that chromaffin cells possess strychnine-sensitive glycine receptors. These findings suggest that glycine is not a universally inhibitory neurotransmitter.

The present study was designed to evaluate the effects of glycine on dopamine release in the striatum, by *in vivo* neurochemical and *in vitro* molecular techniques. Although genes encoding strychnine-sensitive glycine receptors are expressed in many brain areas, it was not known whether cells in the substantia nigra contain mRNA for strychnine-sensitive glycine receptors (Malosio *et al.*, 1991). *In vivo* effects of glycine in supraspinal regions have not been reported. The present study applied *in vivo* microdialysis in conscious, freely-moving rats, in order to determine whether glycine affects endogenous dopamine release and turnover in the striatum, and whether the effects are strychnine-sensitive. By use of reverse transcription followed by polymerase chain reaction (RT-PCR), transcription of the genes encoding two forms of strychnine-sensitive glycine receptor (adult and neonatal) was examined in the striatum and substantia nigra.

Methods

Microdialysis

Male Sprague-Dawley rats (230–250 g; $n = 5-9$ per treatment group) were anaesthetized with sodium pentobarbitone (50 mg kg^{-1}, i.p.). A microdialysis probe (4 mm length, 20 kD cutoff value, CMA/10, BAS/Carnegie Medicine, West Lafayette, IN, U.S.A.) was placed stereotaxically (David-Kopf Instruments, Tujunga, CA, USA; incisor bar 3.2 mm below the interaural line) in the anterior striatum (1.0 mm anterior to bregma, 2.5 mm lateral to midline suture, 6.5 mm ventral to dura; Paxinos & Watson, 1982) and cemented to the skull, as previously described (Pacak *et al.*, 1992). Body temperature was maintained with a heating blanket.

Artificial cerebrospinal fluid (aCSF; NaCl 189 mM, $CaCl_2$ 3.37 mM and KCl 3.9 mM, pH 6.3) was pumped through the dialysis probe (1.0 μl min^{-1}) with a microinjection pump (CMA 100, BAS/Carnegie Medicine, West Lafayette, IN, U.S.A.).

Experiments were performed in conscious, unrestrained animals 20–24 h after probe implantation. The dialysate was collected into polyethylene tubes containing 15 μl EDTA/ethanol (0.02/1%). After two 30 min baseline collections, aCSF containing glycine (Sigma, St. Louis, MO, U.S.A.) at various concentrations (0.02–20 mM) was administered, with or without strychnine (10 μM, Sigma, St. Louis, MO, U.S.A.) in the perfusate.

The dialysate samples were injected directly into a high-performance liquid chromatograph coupled to an electromechanical detector. Separation of the cathecholamines and their metabolites was achieved by reverse phase liquid

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chromatography (Altex Ion Pair Ultrasphere C-18, 5 μm 4.6 mm ID × 250 mm column, No. 235335), with column temperature 30°C. The mobile phase, consisting of 2.1 l of water, 3.2 g 1-heptanesulphonic acid (No 0-3013, Fisher Scientific, Fairlawn, NJ, USA), 0.2 g EDTA (Fisher No. S-311), 16 ml triethylamine (Fisher No. 0-4884), 12 ml 85% phosphoric acid (Fisher No. A-260-500), and 60 ml acetonitrile (No 015-4 Burdick & Jockson, Muskegon, MI, U.S.A.), was pumped at 0.8 ml min^{-1}.

The detection apparatus included an analytical cell (No CB-100, EiCOM, Kitahatacho Fushimi, Kyoto, Japan) and a detector (No 460, Waters, Millipore, Milford, MA, U.S.A.), with oxidation potential 0.64–0.67 V.

Probe recoveries of dopamine, dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) were measured in a 22°C water bath.

Statistical analyses included two-way analysis of variance with one repeated measure. A P value less than 0.01 defined statistical significance.

Transcription of genes encoding for glycine receptors

The existence of mRNA encoding the main adult and neonatal forms of the strychnine-binding subunit of glycine receptors was examined by RT-PCR, using the medulla oblongata as a positive control. The striatum, medulla oblongata, and substantia nigra (SN) of rats were dissected and immediately frozen in liquid nitrogen. Striata and SN of six rats were pooled for RNA extraction. RNA was extracted from the frozen tissues as described by Chomczynski & Sacchi (1987), using a commercial solution (RNAzol B, Tel-Test Inc., Friendswood, TX, U.S.A.). The quality of the RNA preparation was examined by electrophoresis of the RNA on a 16% formaldehyde, 1.25% agarose gel, by visualisation of the 28S and 18S bands. Oligonuleotide primers for the genes were synthesized by Lofstrand Labs, Inc. (Gaithersburg, MD, U.S.A.), according to published sequences (Grenningloh *et al.*, 1987; Kuhse *et al.*, 1990). The sequences of the primers for the adult rat 48K subunit glycine receptor (encompassing a cDNA fragment of 542 base pairs) were (upstream) CTTC-CTGGATAAGCTTATGGGAAGG and (downstream) CTCTTCCTTCAGGATAAACTGAGGC. The primers for the rat neonatal glycine receptor (encompassing a cDNA fragment of 487 basepairs) were (upstream) GCAAAGAC-CATGACTCCAGG and (downstream) GCTGCATT-GTACAGGTCTGG.

Reverse transcription (RT) with the specific downstream primers was performed for 2 h at 42°C, using 5 μg of total RNA, in a total volume of 20 μl, containing 50 mM Tris-HCl (pH = 8.3), 40 mM KCl, 6 mM $MgCl_2$, 1 mM DTT, 40 u RNAsin (Promega, Madison, WI, U.S.A.), 2 μg bovine serum albumin, 2 μM of the downstream primer, 0.2 mM of each deoxynucleotide, and 5 units of AMV reverse transcriptase (Gibco-BRL, Gaithesburg, MD, U.S.A.). Five microliters of the RT product were subjected to 40 cycles of PCR, using the GeneAmp kit (Cetus-Perkin-Elmer, Norwalk, CT, U.S.A.). Each reaction was carried out in a total volume of 100 μl, containing 25 pmol of each oligonucleotide primer and 2.5 u AmpliTaq DNA polymerase. Each cycle consisted of denaturation at 94°C for 1 min, annealing at 55°C for 90 s, and extension at 72°C for 90 s.

After amplification, 10 μl of the product of each reaction was electrophoresed on a 4-20% Tris-glycine gel (Novex, San Diego, CA, U.S.A.). The gel was stained with ethidium bromide, exposed to u.v. light, and photographed.

Results

Basal microdialysate dopamine, DOPAC, and HVA concentrations averaged 13.5 ± (s.e.mean) 0.50, 2067 ± 131, and 994 ± 102 nmol l^{-1} ($n = 20$). *In vitro* recoveries of dopamine, DOPAC and HVA averaged 20 ± 1.6, 22 ± 2 and 26 ± 0.5% ($n = 6$). Applying these recoveries, and correcting for the volume of preservative (15 μl added to 30 μl of microdialysate), estimated extracellular fluid concentrations of dopamine, DOPAC, and HVA were 0.10 ± 0.004, 17.8 ± 0.9, and 5.7 ± 0.6 μmol l^{-1}.

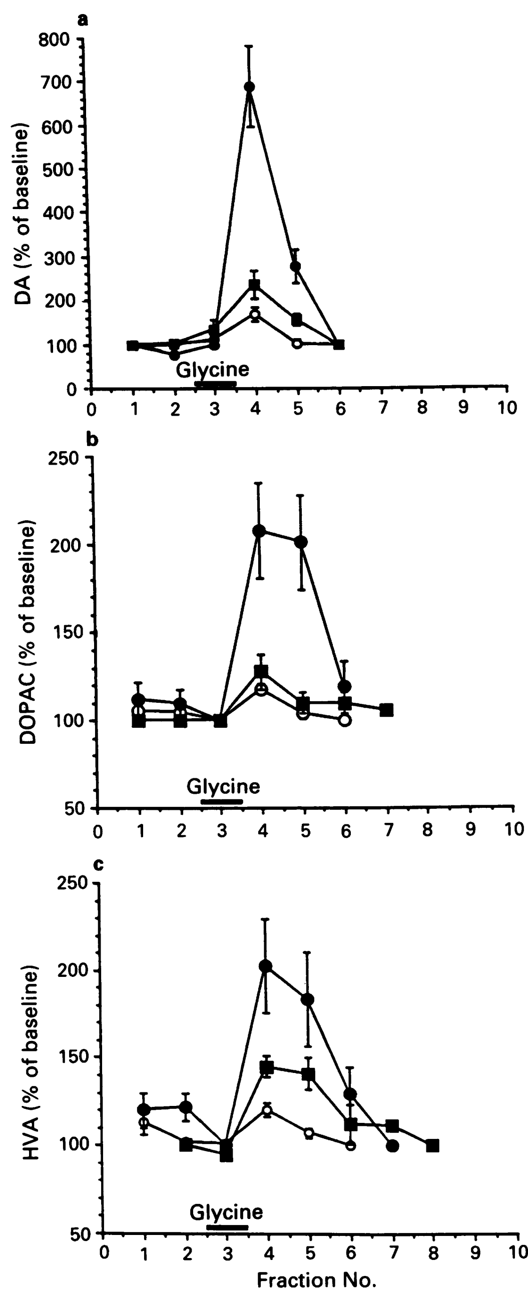

Figure 1 Effects of glycine (0.2 mM, ○; 2 mM, ■; 20 mM, ●) on microdialysate concentrations of dopamine (DA), dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in the corpus striatum of conscious rats. The bars indicate the period of glycine administration. Each fraction corresponds to 30 min.

Glycine dose-dependently increased microdialysate dopamine, DOPAC, and HVA levels (Figures 1,2). Although the approximately 9 fold increase in levels of dopamine above baseline was larger than the approximately 3.5 fold increases in levels of metabolites, the absolute increases in dopamine (about 900 nmol l^{-1}) were far smaller than those in the metabolites (about 8000 nmol l^{-1}). The increment in dopamine appeared mostly in a single fraction following glycine, whereas the elevations in metabolites occurred during both the first and second 30 min collections after glycine.

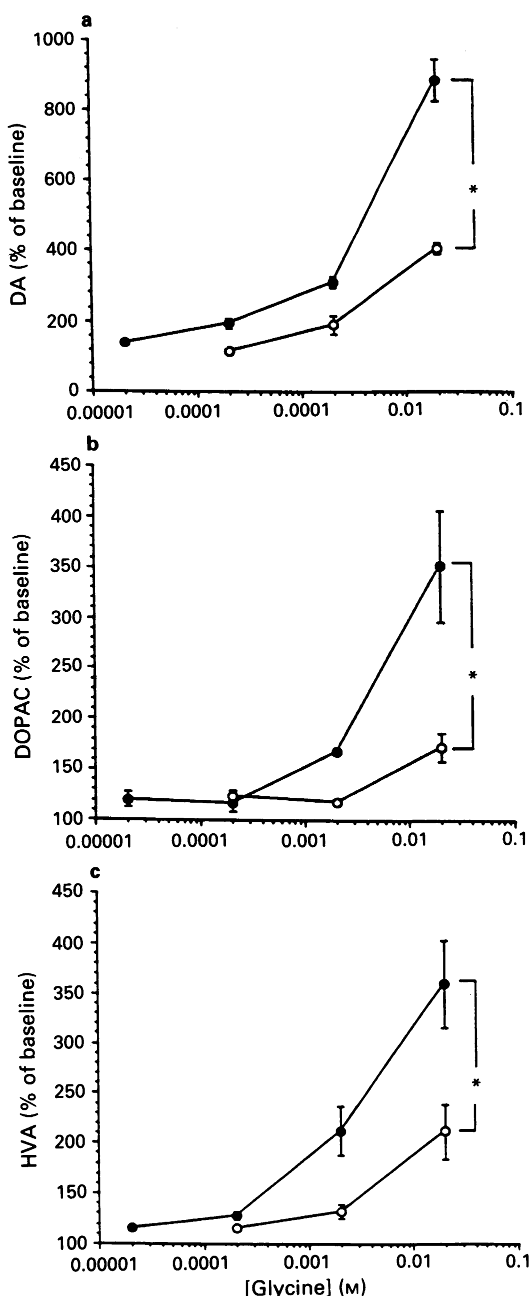

Figure 2 Microdialysate concentrations of dopamine (DA), dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) after glycine administration with (O) or without (●) strychnine (10 μM). $^*P<0.01$.

Strychnine markedly reduced glycine-evoked increases in dialysate dopamine, DOPAC, and HVA levels (Figure 2), shifting the dose-response curves to the right by at least 10 fold.

When striatal RNA was used as a template, we could not detect any RT-PCR product, with primers encompassing a region of the adult form of the glycine receptor, or with those spanning sequences of the neonatal type (Figure 3, lanes 1,4). In contrast, using extracted RNA from SN, a product of the adult glycine receptor, but not the neonatal type could be detected. A single clear band of the expected size (542 and 487 bp for the adult and neonatal types, respectively) was evident in both reactions in which RNA from the medulla, which served as the positive control, was used (Figure 3, lanes 2,5). These bands were evident also after 25 cycles of PCR (data not shown).

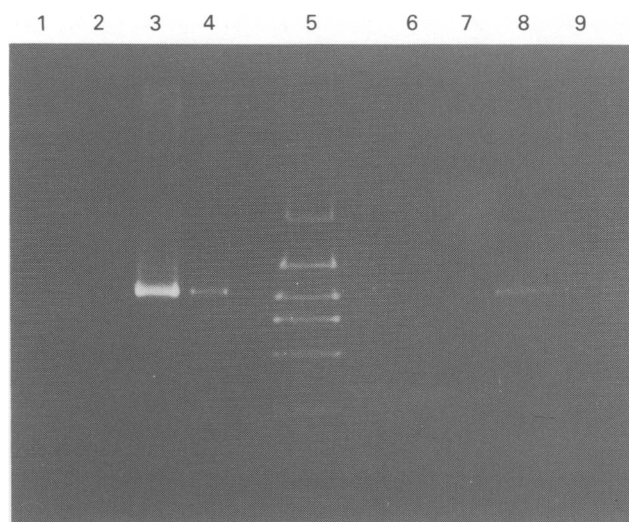

Figure 3 Ethidium bromide stained electrophoresed RT-PCR products of: (lane 1) water blank, amplified with primers encompassing a 542 bp region of the adult-type (48K subunit) glycine receptor; (lane 2) RNA from striatum, with primers encompassing a 542 bp region of the adult-type (48K subunit) glycine receptor; (lane 3) RNA from medulla oblongata, with primers encompassing a 542 bp region of the adult-type (48K subunit) glycine receptor; (lane 4) RNA from the substantia nigra, with primers encompassing a 542 bp region of the adult-type (48K subunit) glycine receptor; (lane 5) gel size markers (band sizes 1000, 700, 500, 400, 300, and 200 bp) (Research Genetics, Hansville, AL, U.S.A.); (lane 6) water blank, with primers encompassing a 487 bp region of the neonatal-type glycine receptor; (lane 7) RNA from striatum, with primers encompassing a 487 bp region of the neonatal-type glycine receptor; (lane 8) RNA from medulla oblongata, with primers encompassing a 487 bp region of the neonatal-type glycine receptor; (lane 9) RNA from the substantia nigra, with primers encompassing a 487 bp region of the neonatal-type glycine receptor.

Discussion

In the present study, glycine administered via a microdialysate probe in the striatum of conscious rats dose-dependently increased dopamine, DOPAC, and HVA concentrations in the dialysate. These results indicate that glycine releases dopamine into the extracellular fluid, with subsequent neuronal reuptake of dopamine and intraneuronal conversion of dopamine to DOPAC and with extraneuronal uptake of dopamine and DOPAC and extraneuronal conversion to HVA. The results therefore indicate that the net effect of glycine in the striatum is to stimulate endogenous dopamine release.

Strychnine attenuated glycine-evoked release of dopamine and its metabolites by at least 90%, suggesting involvement of a strychnine-sensitive receptor. Analogously, glycine stimulates [^{3}H]-noradrenaline release *in vitro* in hippocampal slices (Raiteri *et al.*, 1990; Schmidt & Taylor 1990), stimulates [^{3}H]-acetylcholine release in striatum (Taylor *et al.*, 1988), and stimulates [^{3}H]-dopamine release in ventral tegmentum (Gunglach & Beart., 1982), with all these effects blocked by strychnine.

The concentrations of glycine in the microdialysate that were required to stimulate dopamine release probably substantially exceeded those required to act at striatal effector sites, because the permeable membrane maintains a concentration gradient between the perfusate and the extracellular fluid, and because glycine in the extracellular fluid may be subject to metabolism or cellular uptake before reaching dopaminergic effector sites (Roberts & Anderson, 1979). Thus, administration of glycine directly into the striatum via a cannula attached to the microdialysis probe shifts the glycine concentration-microdialysate dopamine response curve to the left by about 10 fold, compared to the curve

obtained with administration of glycine via the perfusate in the probe (Yadid *et al.*, unpublished observations). At present, the exact relationship between glycine concentrations in the perfusate and endogenous glycine concentrations at striatal effector sites is unclear. The basal glycine concentration in striatal extracellular fluid is about 10 μM (M. Globus, personal communication). When glycine is administered via a cannula attached to the microdialysis probe, the minimum concentration producing significant increments in microdialysate dopamine is about 50 μM, whereas in the present study, the minimum effective concentration of glycine administered via the probe membrane was 200 μM.

Since glycine is thought to be an inhibitory neurotransmitter, one would expect the net stimulatory effect of glycine on striatal dopamine release to occur via an indirect local mechanism. For instance, glycine could inhibit inhibitory interneurones in the striatum; inhibit inhibitory heteroreceptors on dopaminergic terminals; de-inactivate local calcium channels by membrane hypopolarization (Llinas *et al.*, 1983); or increase transmembrane Cl^- conductance in nearby axons (Simmonds, 1983; Raiteri *et al.*, 1990), thereby producing receptor-mediated depolarization rather than hyperpolarization.

Mechanisms requiring the synthesis of strychnine-sensitive glycine receptors within the striatum can be excluded, since we failed to detect expression of genes encoding the two main types of glycine receptor (adult and neonatal types) in the striatum by the highly sensitive method of RT-PCR. Consistent with these results, studies using *in situ* hybridization have reported expression of mRNA encoding strychnine-sensitive glycine receptors in supraspinal structures, but not in the striatum (Malosio *et al.*, 1991). Thus, if glycine releases dopamine via inhibition of local inhibitory mechanisms, the receptors mediating this effect must differ structurally from those already identified or must be associated with axons projecting from sources outside the striatum.

The present results did confirm the expression of mRNA encoding the adult form of strychnine-sensitive glycine receptor in the substantia nigra, which is the source of dopaminergic innervation of the striatum. This result raises the possibility that strychnine-sensitive glycine receptors are transported in the axoplasm from dopaminergic cell bodies in the substantia nigra to dopaminergic terminals in the striatum, and that occupation of the receptors evokes transmitter release from the terminals. Indeed, in substantia nigra zona compacta neurones, glycine was shown to produce a membrane depolarization that was blocked by strychnine (Mercuri *et al.*, 1990). The results therefore suggest a stimulatory effect of what has been thought to be a purely inhibitory neurotransmitter in the central nervous system.

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