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Antagonists of Both D1 and D2 Mammalian Dopamine Receptors Block the Effects of Dopamine on *Helix aspersa* Neurons

Young-Kee Kim* and Michael L. Woodruff[1]

Department of Agricultural Chemistry, Chungbuk National University, Cheongju 360-763, Korea
[1]Department of Anatomy and Cell Biology, UCLA School of Medicine, USA
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Abstract: Dopamine mediates inhibitory responses in *Helix aspersa* neurons from the right parietal lobe ("F-lobe") of the circumoesophageal ganglia. The effects appeared as a dose-dependent hyperpolarization of the plasma membrane and a decrease in the occurrence of spontaneous action potentials. The average hyperpolarization with 5 μM dopamine was −12 mV (± 1.5 mV, S.D., n=12). Dopamine also modulated the currents responsible for shaping the action potentials in these neurons. When dopamine was added and action potentials were triggered by an injection of current, the initial depolarization was slowed, the amplitude and the duration of action potentials were decreased, and the after-hyperpolarization was more pronounced. The amplitude and the duration of action potential were reduced about 15 mV and about 13% by 5 μM dopamine, respectively. The effects of dopamine on the resting membrane potentials and the action potentials of *Helix* neurons were dose-dependent in the concentration range 0.1 μM to 50 μM. In order to show 1) that the effects of dopamine were mediated by dopamine receptors rather than by direct action on ionic channels and 2) which type of dopamine receptor might be responsible for the various effects, we assayed the ability of mammalian dopamine receptor antagonists, SCH-23390 (antagonist of D1 receptor) and spiperone (antagonist of D2 receptor), to block the dopamine-dependent changes. The D1 and D2 antagonists partially inhibited the dopamine-dependent hyperpolarization and the decrease in action potential amplitude. They both completely blocked the decrease in action potential duration and the increase in action potential after-hyperpolarization. The dopamine-induced slowdown of the depolarization in the initial phase of the action potentials was less effected by SCH-23390 and spiperone. From the results we suggest 1) that *Helix* F-lobe neurons may have a single type of dopamine receptor that binds both SCH-23390 and spiperone and 2) that the dopamine receptor of *Helix* F-lobe neurons may be homologous with and primitive to the family of mammalian dopamine receptors.
Key words: Dopamine receptors, *Helix* neurons, neuronal modulation, SCH-23390, spiperone.

Dopamine is a major neurotransmitter in several species of mollusks and appears to modulate ionic channels through receptor activation (Gospe, 1983). The effects of dopamine are excitatory, inhibitory, or biphasic depending on the specific molluscan neuron (Ascher, 1972; Kerkut *et al.*, 1975). In excitatory responses, dopamine decreases K^+ channel activity or increases Ca^{2+} channel activity so that resting membrane potential is depolarized, and the frequency and the duration of action potentials is increased (Paupardin-Tritsch *et al.*, 1985). Dopamine-induced inhibitory responses are usually accompanied by hyperpolarization of the membrane potential and/or decreases in the duration and the amplitude of action potentials. The physiological mechanism of the inhibitory effects may include an increase in K^+ channel activity or a decrease in Ca^{2+} channel activity (Harris-Warrick *et al.*, 1988).

Although the effects of dopamine have been intensively investigated, the dopamine receptors in molluscan neurons are poorly understood. Dopamine receptors have been characterized from vertebrate tissues and classified into two major subtypes, D1 and D2. These subtypes are distinguished by the receptor-mediated activation of different intracellular second messenger systems as well as by pharmacological criteria. Recently more detailed classification has revealed five subtypes by structural analysis of receptor genes (Civelli *et al.*, 1991; Sibley *et al.*, 1992). D1 receptors stimulate cyclic AMP-dependent protein phosphorylations through the

*To whom correspondence should be addressed.
Tel: 82-431-61-2560, Fax: 82-431-69-2549.

activation of adenylate cyclase. However, they may also be linked to diverse signaling pathways other than the cyclic AMP system (Kebabian *et al.*, 1979; Niznik, 1987). D2 receptors connect to several signal transduction pathways but are most often linked to an inhibition of adenylate cyclase or inhibition of phospholipase C (Niznik, 1987; Fischer *et al.*, 1987). Purification and functional reconstitution of D1 receptor (Dearry *et al.*, 1990; Zhou *et al.*, 1990; Sunahara *et al.*, 1990) and D2 receptor (Bunzow *et al.*, 1988) have been accomplished.

Vlieger *et al.* (1986) found that the dopamine-induced hyperpolarization of neurons from mollusc *Lymnaea stagnalis* was inhibited by D2 antagonist, (−)sulpiride and cyclic AMP analogs. They concluded that the dopamine receptors of *Lymnaea* resemble the D2 type of vertebrate receptors. In this report we analyzed the effects of antagonists of both D1 and D2 receptors on dopamine-dependent inhibition in *Helix aspersa* neurons. We found that dopamine-induced inhibition is blocked by both types of receptor antagonists. The qualitative and quantative similarities of the effects of these two antagonists on the several electrophysiological characterics of these neurons are consistent with a model where there may be only one type of dopamine receptor in these neurons.

Materials and Methods

Animals

Snails (*Helix aspersa*) were purchased from Connecticut Valley Biologicals (Stamford, USA) and were maintained in a state of hibernation until they were used in the experiments.

Chemicals

Antagonists SCH-23390, hydrochloride {R(+)-8-Chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1-H-3-benzazepin-7-ol HCl}, and spiperone, {8-[4-(4-Fluorophenyl)-4-oxobutyl]-1-phenyl-1,3,8-triazaspiro-[4,5]decan-4-one}, were purchased from Research Organic Inc. (Natick, USA). Dopamine, ascorbic acid, and the general chemicals were purchased from Sigma Chemical Co. (St. Louis, USA).

Dissection

Snails were wetted for two hours before the beginning of dissection so that they would become active. They were sacrificed by severing the neck just below the circumoesophageal ganglion. The ganglion from each snail was removed and pinned to cured Sylgard in a 35 mm plastic petri dish. The ganglia were incubated in normal *Helix* Ringer's solution: 80 mM NaCl, 4 mM KCl, 7 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1 mM $NaHCO_3$, 10 mM Glucose, and 10 mM HEPES, pH 7.4. The ganglia were exposed to trypsin (2.5 mg/ml; in normal *Helix* Ringer's solution) for 15 mins at room temperature to digest the connective tissue sheath that covers the neurons. Trypsin was washed out with 500 ml of Ringer's solution and the outer connective tissue sheath and inner sheath were removed by microdissection.

Cell identification

Many neurons of the *Helix* ganglion have been identified and characterized by Kerkut *et al.* (1975). The experiments in this report used neurons F-14 to F-16 and F-28 to F-32 which are clustered together in the right parietal lobe of the subesophageal ganglion. We tentatively identified the neurons based on the location map of Kerkut *et al.* They are fairly easy to recognize by their location and size. The cells are situated next to the giant neurons F-1 and F-2 in a lateral to dorsolateral position and have a diameter of approximately 50 μm. The neurons were also identified by action potential beating frequency and action potential shape.

Electrophysiology

Neurons were penetrated by a hollow glass intracellular pipette filled with 3 M KCl (resistance 5~10 MΩ). After the resting potential stabilized and the beating rate of action potential became regular, the effects of dopamine, before and after receptor antagonists were added, were determined. Dopamine was added directly to the bath by gently pipetting and mixing a concentrated solution of dopamine from a Gilson pipetman. The effects of dopamine on the resting membrane potential were most clearly demonstrated by suppressing the beating activity of the neurons by injecting just enough current so that the membrane hyperpolarized by 2~3 mV. The effect of dopamine on action potential shapes was determined in a similar fashion. After hyperpolarizing the membrane by 2 to 3 mV to prevent spontaneous activity, action potentials were triggered by injecting depolarizing currents (0.5~1 nA) from the artificially hyperpolarized potential. Using this procedure the action potentials were very reproducible from stimulus to stimulus and it allowed us to assay subtle dopamine-dependent changes in action potential shape. The effects of dopamine on resting potential and action potential are similar qualitatively to the effects observed without the small induced hyperpolarization (Tiwari *et al.*, 1992). Stimulus pulses were removed from the records during analysis by subtracting pulse-only traces which were recorded from the same cell right after recording each action potential. Pulse-only traces were obtained

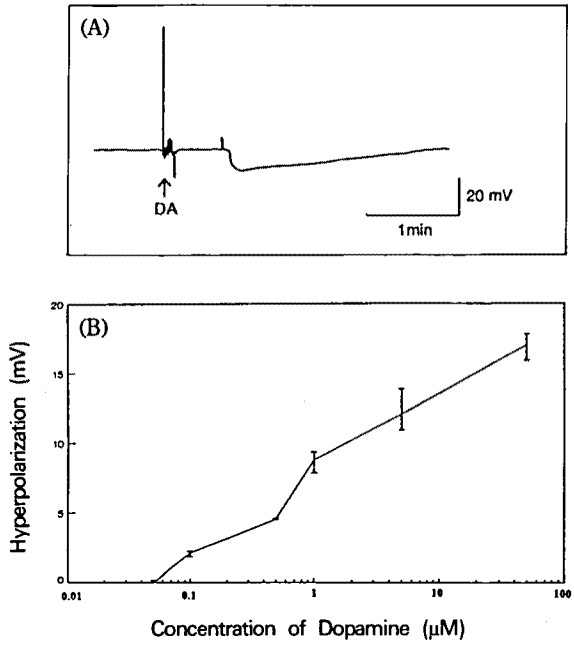

Fig. 1. Effect of dopamine on the membrane potential of F-lobe neurons. (A) Dopamine-induced hyperpolarization. Dopamine (final conc. 5 μM) was added to the edge of the petri dish (3.5 cm diam.) containing *Helix* ganglia. During the application of dopamine one action potential was triggered with an upward spike of 65 mV. (n=12) (B) Dopamine-induced hyperpolarizations with different concentrations of dopamine between 0.01 μM and 50 μM. N≥2~5 at each concentration.

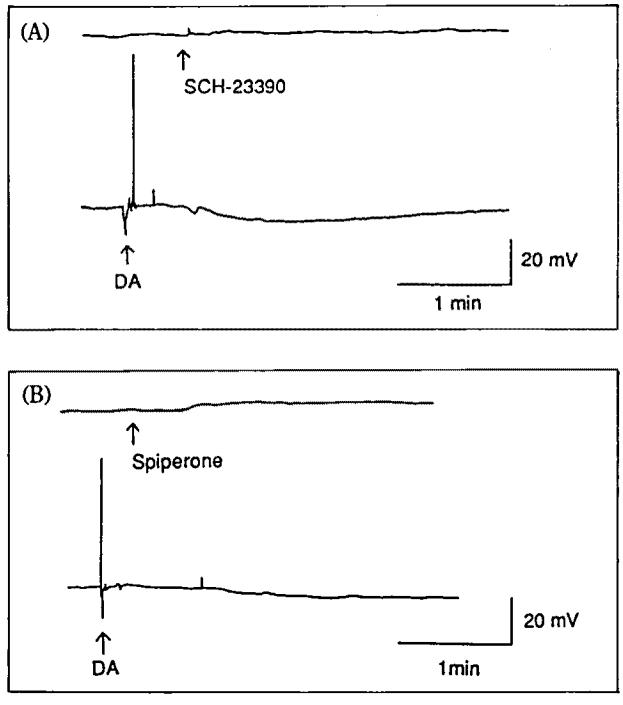

Fig. 2. Antagonists of mammalian dopamine receptors inhibit the effect of dopamine. (A) Upper trace: The effect of D1 antagonist, SCH-23390 (1 μM) itself on membrane potential. Lower trace: The effect of additional application of dopamine (5 μM). (n=5) (B) Upper trace: The effect of D2 antagonist, spiperone (0.4 nM) itself on membrane potential. Lower trace: The effect of Dopamine (5 μM) at 5 min after spiperone treatment (n=6).

by hyperpolarizing the membrane by an additional 5 mV-just enough to prevent action potential firing. An IBM-AT computer and Analog to Digital board (Axon Instrument Co., Foster City, USA) were used to record action potentials. An intracellular amplifier (KS-700, WPI, Sarasota, USA) was used to amplify signals from the cell. All the signals were recorded on a video type using a Sony VHS digitized system and monitored during the experiment with a storage oscilloscope (Hitachi V-134).

Drug effects

Dopamine was added to the bath from a concentrated stock solution containing 0.1 mM ascorbate as an antioxidant. A control solution with only ascorbate did not show any physiological effects on the neurons. SCH-23390 and spiperone were dissolved in the normal *Helix* Ringer's solution from a concentrated ethanol stock solution. The final concentration of ethanol was less than 0.5%. Ethanol at this concentration had no effect on neuronal activity.

Results

Dopamine effects on membrane potential

The measured resting potentials of the right parietal neurons F-14, F-15, F-16, F-28, F-29, F-30, F-31, and F-32 were from −50 mV to −60 mV in *Helix* Ringer's solution. These neurons had spontaneous action potentials as an intermittent beating activity. A bath application of dopamine caused a hyperpolarization of the resting potential and a cessation of spontaneous spiking behavior. Fig. 1A shows the effect of 5 μM dopamine on the membrane potential. An action potential occurred as the pipette with dopamine entered the bath and then as dopamine was slowly added and mixed in the bath. A rapid hyperpolarization of 15 mV was observed, followed by a slow depolarization to near the pretreatment level. The magnitude of the hyperpolarization was dose-dependent between 0.1 μM and 50 μM (Fig. 1B). If 5 μM dopamine is added, the membrane potential hyperpolarizes and then relaxes to near the original membrane potential. The maximal hyperpolarization was about 20 mV, to a potential near the potassium equilibrium potential (E_K=−71 mV).

SCH-23390 and spiperone inhibit the effect of dopamine on membrane potential

Fig. 2 shows that D1 antagonist, SCH-23390, and D2 antagonist, spiperone, both inhibit the hyperpolarizing effect of dopamine in the right parietal neurons.

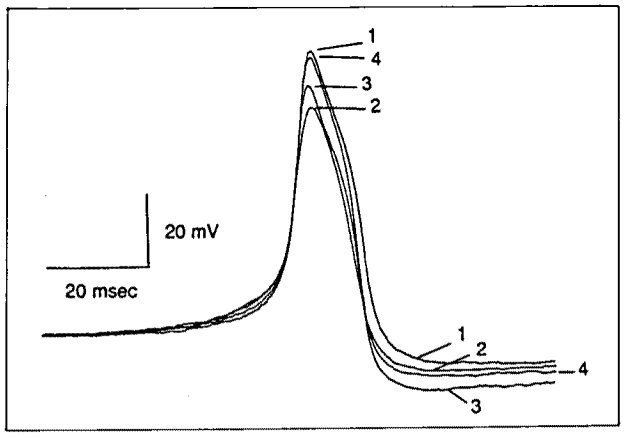

Fig. 3. Dopamine-dependent changes of action potential shape. Action potentials were triggered at different time periods, before (trace-1), and after dopamine addition (trace-2; 1 min after, 3; 10 min after), and after washing out dopamine from the bath (trace-4). They were superimposed at +40 mV of early depolarizing phase of each action potential (n>10).

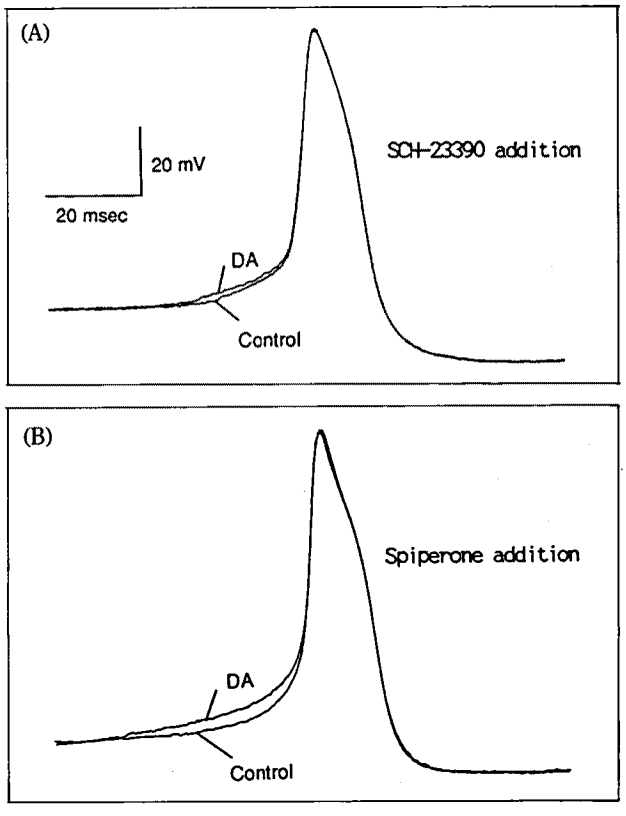

Fig. 4. Effect of antagonists on action potential. (A) An action potential was triggered before antagonist addition (Control) and then SCH-23390 (1 μM) was added. Dopamine (5 μM) was added and an action potential was triggered (DA). These action potentials were superimposed at +40 mV of early depolarizing phase of each action potential (n=5). (B) An action potential was triggered before spiperone treatment (Control) and then spiperone (0.4 nM) was added. No changes were observed with spiperone itself. Another action potential (DA) was triggered after the addition of dopamine (5 μM) (n=6).

The first trace in Fig. 2A shows that SCH-23390 (1 μM) itself has little or no effect on the membrane potential. Addition of 5 μM dopamine shortly after the addition of SCH-23390 induced a small hyperpolarization (Lower trace): much slower and shallower than the hyperpolarization observed without the antagonist. After the addition of SCH-23390, 5 μM dopamine had an average effect normally observed for 0.5 μM dopamine. A complete concentration curve was not determined but it appeared that 1 μM of the D1 antagonist reduced the sensitivity of the cells to doapmine by about 10-fold. Higher concentrations of the antagonists were not used in these experiments. As we will show below, 1 μM antagonist was sufficient to block completely the effect of dopamine on neuronal action potentials.

Like SCH-23390, D2 antagonist, spiperone, had no effect on the resting membrane potential (Fig. 2B, Upper trace), but it inhibited dopamine-induced hyperpolarization. The lower trace of Fig. 2B shows that the effect of 5 μM dopamine was almost completely blocked by spiperone. The dopamine-induced hyperpolarizations after spiperone addition were reduced 90 to 100% of the control values to a level normally observed with 0.1 to 0.5 μM dopamine.

Dopamine effects on action potential shape

Voltage-sensitive ion channels that generate and shape the action potentials in the right-parietal neurons are also modulated by dopamine. An action potential generated in normal Ringer's solution is shown in Fig. 3, trace 1. The rapid depolarization is caused by the activation of voltage-dependent Na^+ and Ca^{2+} currents. The repolarization occurs because of the inactivation of these currents and the delayed activation of a voltage-dependent, TEA-sensitive, K^+ current. The right parietal neurons that we used in this study had a prominent shoulder on the repolarizing phase of the action potentials. This is mediated by a Ca^{2+} current which turns off slowly and is similar in ionic mechanism to the shoulder on the falling phase of cardiac action potentials. This prolonged Ca^{2+} current increases the duration of the action potential, which is generally 15 to 20 msec (measured at half-height), and limits the extent of the after-hyperpolarization.

Trace 2 of Fig. 3 shows the change in the shape of the action potential after 1 min of 5 μM dopamine treatment. The rising phase of the initial depolarization is slower, the amplitude of the action potential is reduced by almost 20 mV, the duration of the action potential is reduced by 3 to 5 msec and the after-hyperpolarization is increased proportionately. Ten minutes after the addition of dopamine (trace 3) the action poten-

tial amplitude increased back toward the control amplitude; however, the duration was decreased further and the after-hyperpolarization became deeper. These modulating effects of dopamine were reversible; when dopamine was removed from the bath, the action potentials returned slowly to the control shape (trace 4, Fig. 3). The complex time-dependent changes after the addition of dopamine suggest that more than one voltage-dependent conductance is modulated by dopamine. In a subsequent report we will show that some of the effects of dopamine on the action potential shape can be explained by a dopamine-dependent, reversible decrease in inward Ca^{2+} current.

SCH-23390 and spiperone inhibit dopamine-dependent changes in action potentials

If SCH-23390 and spiperone attenuate the dopamine-dependent hyperpolarization of the right parietal neurons by blocking dopamine binding to dopamine receptors, then they should also inhibit the modulating effects of dopamine on action potential currents. Fig. 4A shows that D1 antagonist, SCH-23390, completely blocks the dopamine-induced decrease in action potential duration and increase in after-hyperpolarization. The only intact effects of dopamine after SCH-23390 treatment were first, a clearly decreased rate of depolarization on the initial rising phase of the action potential and second, a small, less than 5 mV, decrease in the height of the action potential. Quantitatively, the effect of dopamine on the action potential amplitude was much reduced, less than 10% of the effect obtained without SCH-23390, and was not significant on action potential propagation or signaling. SCH-23390 itself had no effect on action potential shape.

A nearly identical effect was observed with D2 antagonist, spiperone, (Fig. 4B). Spiperone (0.4 nM) itself had no modulating effect on the action potential shape; however, the changes normally observed with dopamine were blocked after spiperone addition. Again the only intact dopamine effects were a less sharp rise in the initial rising phase of the action potential and a small decrease in action potential amplitude. This latter effect, like that observed with the D1 antagonist, was much reduced from the normal dopamine effect.

Discussion

Effect of dopamine on the resting and action potentials

The effect of dopamine in modulating ionic channels is believed to operate by a receptor-activated second messenger system and the results presented here provide additional support for that hypothesis. All the effects of dopamine represent inhibitory actions; hyperpolarization, reduced frequency, amplitude, and duration of action potential, and increase after-hyperpolarization. The hyperpolarization induced by dopamine in these neurons suggests that dopamine may affect leakage conductances that are important in determining the resting membrane potential. An increase in K^+ leakage conductance or a decrease in Cl^- permeability of the membrane could cause the hyperpolarization. We at tempted to determine whether there was an increase or decrease in input resistance of membrane after dopamine treatment, but obtained varying results. Perhaps dopamine decreases inward current and increases outward current to effect a hyperpolarization. The slow return of the potential to near the pretreatment level, even though dopamine is not removed from the bath, may be due to biochemical desensitization in the signaling pathway which results in a voltage-dependent removal of inactivation of the ion channels.

Two dopamine-dependent changes in the shape of action potentials were also transient: an initial slowing of the depolarization toward the firing threshold and a decrease in the action potential amplitude. All three of these phenomena, hyperpolarization, slowed depolarization, and reduced amplitude, may be effected by a dopamine-dependent increase in K^+ conductance. There may be more than one type of K^+ conductances important in these changes, a leakage K^+ conductance and a voltage-dependent K^+ conductance sensitive to tetraethylammonium (TEA) inhibition. When TEA was added to the bath, the dopamine-dependent decrease in initial depolarization rate was completely absent; however, there was no change in the effect of dopamine on the resting membrane potential (Kim and Woodruff, manuscript in preparation).

The other effects of dopamine on action potential shape, decreased duration and increased after-hyperpolarization, appeared monotonically, and that was without any apparent negative feedback or tendency to reverse. The ionic mechanisms for these changes, however, are probably complex and involve more than one type of ion channel.

Effect of dopamine antagonists

The antagonists either completely inhibited the effects of dopamine (e.g. decrease in action potential duration and increase in after-hyperpolarization) or quantitatively reduced the effects of dopamine (e.g. decrease in hyperpolarization and the action potential amplitude). If there is a single population of the dopamine receptor in the membrane which interact with only one type of effector molecule (e.g. G-protein) to affect different ion channels, it is difficult to model a complete

and partial block for the different functions. If a block of the dopamine-induced activity is reduced by 25% to 50% for one function, then it should be reduced in that range for the other functions as well. If, on the other hand, there are two subtypes of dopamine receptors or two subtypes of interacting effector molecules, the results are more easily modeled. Since both antagonists have similar relative effects, the latter possibility, a single dopamine receptor with more than two effector molecules, seems more likely. Activation of two separate effector pathways, and therefore different controllable pathways, could also explain why some of the dopamine-dependent changes show reversal while others do not. Dopamine-induced hyperpolarization, slowed depolarization and reduced amplitude are all reversed in the presence of dopamine (desensitization), whereas the action potential duration and after-hyperpolarization effects are not reversed in parallel.

The results shown in this study provide a clue to understanding the functional structure of the dopamine receptor. SCH-23390 only blocks D1 type dopamine receptors in vertebrate tissue and spiperone only blocks vertebrate D2 type dopamine receptors. In *Helix* neurons both vertebrate receptor antagonists inhibit various dopamine-dependent changes. The qualitative and quantitative similarities in the block by SCH-23390 and spiperone suggest that there is a single dopamine receptor type in the snail neurons and, from the arguments presented above, that this receptor is capable of interaction with two or more effector molecules in the membrane. The dopamine receptor of these neurons may be homologous to the vertebrate dopamine receptors and it may represent an evolutionary earlier form of the molecule-one more broad in its ability to bind antagonists and, perhaps, to interact with second messenger activating systems. Biochemical and structural analysis of the snail dopamine receptor should supply insight into the requirements for SCH-23390 and spiperone binding capability and how these binding sites effect dopamine interaction with dopamine receptors.

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