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Effects of Dopamine Agonists on Primary Cultured Neurons from Various Brain Regions

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Abstract—Using 2 to 4 day-old postnatal rats, primary brain cell cultures were made from various brain regions (substantia nigra, hippocampus, striatum, and nucleus accumbens). Whole-cell patch clamp technique was used for electrophysiological studies. Neurons cultured from substantia nigra were characterized more in detail to test whether these cultured neurons were appropriate for physiological studies. Immunocytochemical and electrophysiological properties of these cultured neurons agreed with those from other *in vivo* or *in vitro* studies suggesting that cultured neurons maintained normal cytological and physiological conditions. Modulation of ionic channels through dopamine receptors were studied from brain areas where dopamine plays important roles on brain functions. When neurons were clamped near resting membrane potential (−74 mV), R(+)-SKF 38393, a specific D_1 receptor agonist, activated cultured striatal neurons, and dopamine itself produced biphasic responses. Responses of cultured hippocampal neurons to dopamine agonists were kinds of mirror images to those from striatal neurons; D_1 receptor agonists inhibited hippocampal neurons but quinpirole, a D_2 receptor agonist, activated them. Neurons cultured from nucleus accumbens were inhibited by dopamine.

Keywords □ dopamine receptors, cultured brain neurons, striatum, hippocampus, nucleus accumbens.

So far, most of *in vitro* electrophysiological studies of the vertebrate brain have been conducted using brain slice preparation. However, in slice preparations, it is difficult to investigate the authentic functions of specific receptors and ion channels because neurons have complicate synaptic connections with adjacent neurons. Furthermore, slice preparations are not adequate to apply patch clamp technique, one of the most salient progresses in recent electrophysiology (Hamil *et al.*, 1981).

With these reasons, many investigators have been interested in the development of neuronal culturing techniques. The application of this technique yet has been limited because of the difficulties of culturing from postnatal animals, and cultured brain cells used by other researchers have mostly been derived from the embryonic brain (Chiodo and Kapatos, 1987; Di porzio *et al.*, 1987). In embryonic brain, however, brain structures are not formed yet, and cultures were usually obtained from unspecified brain regions. In addition, experimental results could be misinterpreted because the signal transduction components have not been completed.

We have reported the establishment of special technique of making primary brain cell cultures from identified brain nuclei (substantia nigra and hippocampus) of the postnatal animals (Kim *et al.*, 1990; Brown *et al.*, 1993). In this study, culturing technique was expanded to other brain regions, ventral striatum and nucleus accumbens, and patch clamp technique was applied on cultured neurons to investigate physiological roles of dopamine receptors. Brain areas in which we were interested were substantia nigra, the ventral striatum, hippocampus, and nucleus accumbens. Dopaminergic nervous systems in these brain regions are known to be involved in the etiology of motor and mental diseases such as Parkinsonism, schizophrenia, and drug abuse (Hornykiewicz, 1963; Snyder, 1973; Stevens, 1979; Defrance, 1985; Wise, 1987). In this paper, effects of dopamine agonists on cultured neurons from those brain regions are discussed in aspects of their physiological importance in brain functions.

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Materials and Methods

Cell Culture

Using 2 to 4 day-old postnatal Long Evans rats, primary neuronal cultures were made from several brain regions (substantia nigra pars compacta and pars reticulata, ventral striatum, CA1 and CA3 regions of hippocampus, and nucleus accumbens). Briefly, brains were removed and immersed into the osmotically balanced solution (130 mM NaCl; 4.5 mM KCl; 2 mM $CaCl_2$; 33 mM D-glucose; and 5 mM PIPES buffer, pH 7.4). After making slices (400 μm thick) with a vibratome (Lancer 1000), tissue fragments containing specific brain nuclei were dissected out. Brain tissues were incubated for 30 min in a papain solution (12 units/m*l*), and then were dissociated with a pasteur pipette in a culture medium.

Before plating neuronal cells, the bottom of the modified dish was coated with collagen and a feeder layer consisting of non-neuronal cells. To suppress overgrowth of non-neuronal cells, the feeder layer was treated with antimitotic agents, 5′-fluoro-2′-deoxyuridine (15 mg/m*l*) and uridine (35 mg/m*l*). Cultures were kept at 37℃ with saturated humidity, in an atmosphere of 10% CO_2 and 90% air.

Electrophysiology

The whole-cell patch clamp technique was employed for electrophysiological studies. A patch clamp amplifier (List EPC7) was used for the voltage clamp experiments. The standard internal solution contained 120 mM K-aspartate, 40 mM NaCl, 5 mM HEPES/KOH buffer, 0.5 mM EGTA/KOH, 0.25 mM $CaCl_2$, 2 mM $MgCl_2$, 2 mM Na_2ATP and 100 M Na_2GTP, 5~6 mM KOH, pH 7.2. Throughout the experiment, cells were superfused with an oxygenated krebs solution containing 146 mM NaCl, 5 mM KCl, 2.4 mM $CaCl_2$, 1.3 mM $MgCl_2$, 5 mM HEPES/NaOH buffer, 11 mM D-glucose, and 0.5 to 1 μM tetrodotoxin (Calbiochem., La Jolla, CA). The cells were clamped at −74 mV and experimental temperature was maintained around 31℃.

Dopamine (Sigma), SKF38393 (RBI, Natrich, MA), and quinpirole (RBI) were used as dopamine agonists. Drugs were applied by pressure ejection from glass pipettes (tip diameter is about 7 μm) located around 40 to 50 μm from the soma. To prevent oxidation, dopamine was prepared daily by dissolving it in solution containing *l*-ascorbic acid, and then gassing it with nitrogen.

Reproducible responses were obtained from neurons cultured longer than 2 weeks, and electrophysiological studies on dopamine and GABA receptors were usually conducted on neurons cultured between 3 to 4 weeks.

Immunocytochemistry

Indirect fluorescence immunocytochemical method was used. Cultures were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.12 M phosphate buffer (pH 7.4) for 2 hours at 4℃. After washing with PBS, cultures were freeze-thawed, then treated with 1 M ethanolamine (sigma) for 1 hour at room temperature. To decrease background noise, cultures were incubated in 10% normal goat serum (Organon Teknika Corp., West Chester, PA) for 30 minutes at room temperature. Cultures were double labelled with a mouse monoclonal antibody to tyrosine hydroxylase and rabbit antiserum to GABA (INCSTAR, Stillwater, Minnesota) by incubating cultures for 2 to 3 hours at room temperature. After washing cultures overnight with PBS, they were incubated with secondary antibodies for 2 to 3 hours at room temperature. Goat rhodamine-labelled anti-mouse IgG (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD; Organon Terknika Corp.) and goat fluorescein-labelled antirabbit IgG (Kirkegaard and Perry Laboratories, Inc.) were used as secondary antibodies for TH and GABA antibodies, respectively.

Results and Discussion

Immunocytochemical and Electrophysiological Characterization of Cultured Substantia Nigra Neurons

Substantia nigra is known to be composed of relatively homogeneous neuronal populations, dopaminergic neurons and GABAergic neurons (Parent *et al.*, 1983; Karabelas and Moschovakis, 1985). With these reasons, in this study, substantia nigra was selected for the immunocytochemical characterization of cultured neurons. Examples of dopaminergic and GABAergic neurons are shown in Fig. 1 and Fig. 2. Usually dopaminergic neurons possessed relatively thick three to four processes, meanwhile thinner and more branched processes were found from GABAergic neurons (Kim *et al.*, 1991; Masuko *et al.*, 1992).

GABA is one of the well established neurotransmitters in the substantia nigra. Substantia nigra pars reticulata receives GABA inputs from the striatum (Graybiel and Ragsdale, 1979) and in turn it sends out GABA outputs to the ventromedial thalamic nucleus (Uno *et al.*, 1978). Effects of GABA agonists on cultured substantia nigra neurons are shown in Fig. 3. The cell was clamped at −74 mV and a square-wave depolarizing pulse (20 mV, 100 ms) followed by a 100 ms pause and a hyperpolarizing pulses (50 mV, 100 ms) were ap-

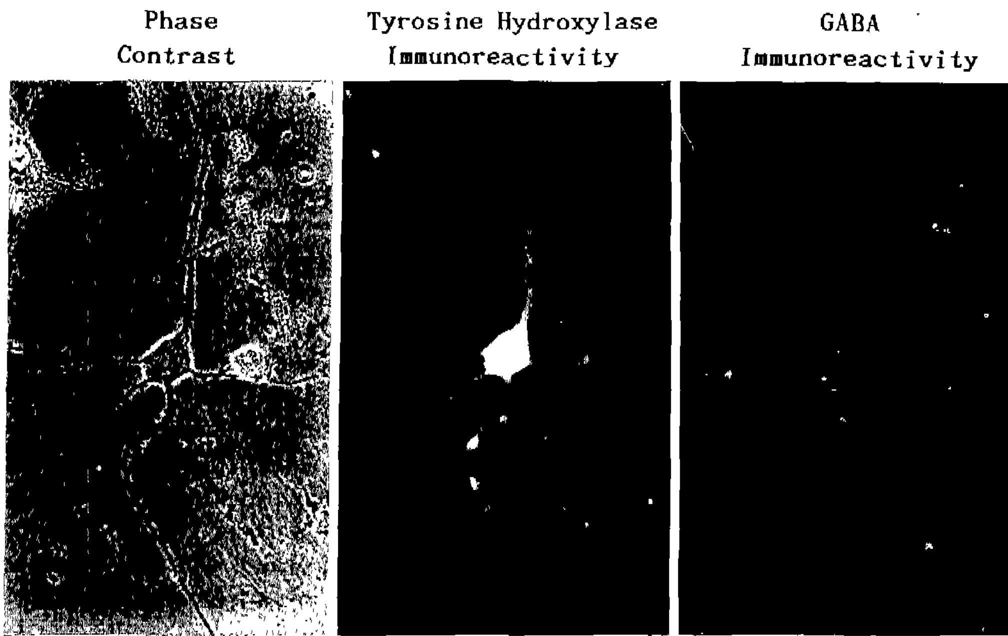


Fig. 1. A typical dopaminergic neuron cultured from the substantia nigra pars compacta.
A substantia nigra pars compacta neuron cultured for 20 days was used. Cultures were fixed and were labelled with antibodies against tyrosine hydroxylase and GABA. Three photographs were taken from the same place. The photograph shown on the left-hand side was taken with phase contrast, the middle photograph shows immunoreactivity to tyrosine hydroxylase (dopaminergic neuron if it is positive), and the photograph on the right-hand side shows immunoreactivity to GABA.

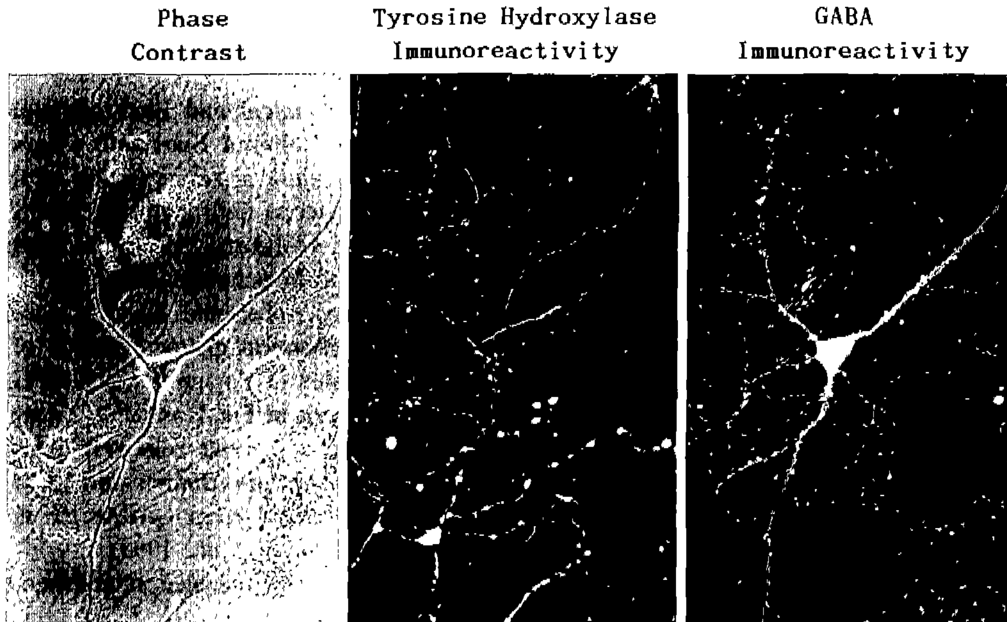


Fig. 2. A typical GABAergic neuron cultured from the substantia nigra pars reticulata.
A substantia nigra pars reticulata neuron cultured for 21 days was used. Small intense spots on the periphery of the soma or on the processes probably represent synaptic buttons from adjacent neurons.

plied every 3 second to monitor the membrane conductance changes. Vertical lines show current pulses evoked by applied voltage commands, and changes in the magnitude of current pulses represent changes in membrane conductance. Upward shift of membrane holding current means that an outward current (hyperpolarization in current-clamp mode) is induced, that is, inhibition of neuronal activity.

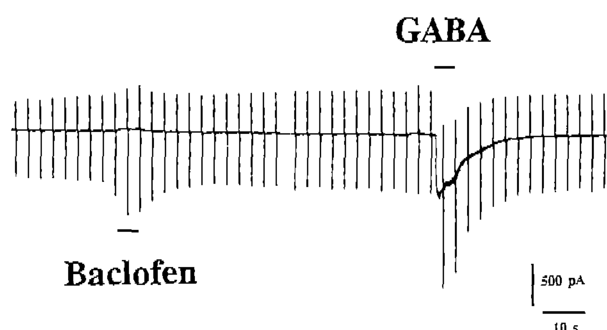


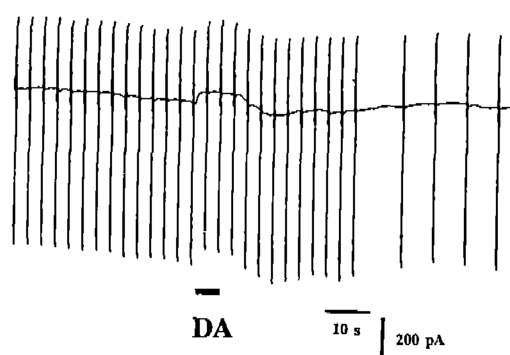
Fig. 3. Effects of GABA agonists on a cultured neuron from the substantia nigra.
Under the experimental conditions employed here, the reversal potentials for chloride and potassium ions were −33 mV and −84 mV, respectively. From the holding potential of −74 mV, depolarizing pulses (20 mV, 100 ms) followed by an interval (0 mV, 100 ms), and then hyperpolarizing pulses (−50 mV, 100 ms) were applied to the cell every 3 sec to monitor the membrane conductance changes. The concentrations of GABA and baclofen were 10 μM and 5 μM, respectively.

Baclofen, a $GABA_B$ agonist, produced an increase in the membrane conductance concomitant with an outward current, suggesting that $GABA_B$ receptors are coupled to the potassium channels. GABA produced an inward current probably by increasing the chloride channel conductance through $GABA_A$ receptors. GABAB receptors also should have been activated by GABA but their actions were probably overshadowed by huge chloride current.

Immunocytochemical and electrophysiological properties of cultured neurons from the substantia nigra agreed with those from other *in vivo* or *in vitro* studies. These results suggested that cultured neurons maintained normal cytological and physiological components.

D_1 Agonist Activates and D_2 Agonist Inhibits Cultured Striatal Neurons

Modulation of ionic channels through D_2 receptors has been extensively studied from various preparations. D_2 agonists increase potassium channel conductance in prolactinoma cells, lactotrophs, and substantia nigra neurons (Israel *et al.*, 1985; Gregerson *et al.*, 1989; Lacey *et al.*, 1987). Specifically, inward rectifying potassium channels (Kim *et al.*, 1990) and pertussis toxin-sensitive G protein are involved in this process (Innis and Aghajanian, 1987; Kim *et al.*, 1990). On the other hand, electrophysiological roles of D_1 receptors are not well established as those of D_2 receptors. It was reported that potassium channel conductance was decreased through D_1 receptors in the substantia nigra pars reticulata neurons (Kim *et al.*, 1990). Also voltage-gated calcium channels were reported to be activated through D_1 receptors (Artalejo *et al.*, 1990).



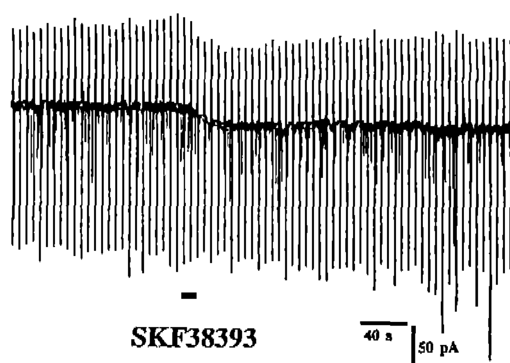


Fig. 4. Effects of dopamine agonists on cultured striatal neurons.
Striatal neurons cultured about 4 weeks were used. The experimental conditions were the same as those in Fig. 3. The concentrations of dopamine and SKF38393 were 10 μM and 20 μM, respectively.

Effects of dopamine agonists on striatal neurons are not clear. Only few ambiguous reports are available yet (Akaike *et al.*, 1987; Calabresi *et al.*, 1987). Interestingly, in the striatum, connections with input and output structures, distribution of dopamine receptors and certain enzymes, and morphological appearances are known to be compartment-specific (for review, see Graybiel, 1990). This compartment-specific distribution of dopamine receptors might provide a partial explanation for the uncertainty of functional roles of dopamine receptors in the striatum.

In this study, D_1 and D_2 receptors seem to be located on the same striatal neuron exerting opposite responses. D_1 agonist activated striatal neurons (Fig. 4A2,

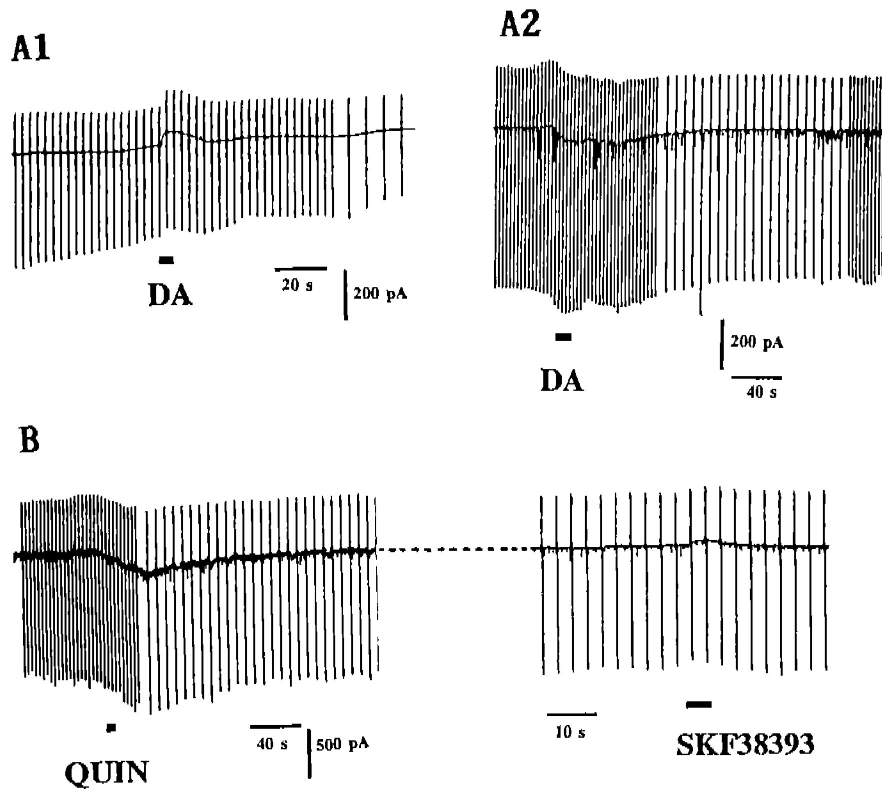


Fig. 5. Effects of dopamine agonists on cultured hippocampal neurons.
Hippocampal neurons (CA1 and CA3), cultured 30 days, were used. Tissue fragments from CA1 and CA3 were mixed together and plated on modified petri dish. Experimental conditions were the same as those in Fig. 3. Concentrations of dopamine agonists used were as follows: dopamine, 10 μM; SKF38393, 20 μM; quinpirole, 1 μM.

inward currents were induced) but dopamine itself produced biphasic responses (Fig. 4A1). Therefore, even though observed from different striatal neurons, these results suggested that D_1 agonist activated striatal neurons by eliciting an inward current and D_2 agonists inhibited them. Neither specific D_1 agonist nor dopamine affected the membrane conductance significantly but they shifted holding currents, probably because potassium channels were not involved in these processes or more than one ionic channels were affected by dopamine agonists.

Effects of Dopamine Agonists on Hippocampal Neurons are Mirror Images to Those on Striatal Neurons

Effects of dopamine agonists on neurons cultured from CA1/CA3 regions of hippocampus were opposite to those from striatum (Fig. 5). D_1 agonist inhibited but D_2 agonist activated them (Fig. 5B). Dopamine produced an outward current in some neurons (Fig. 5A1) or an inward current from others (Fig. 5A2) suggesting that only single population of dopamine receptors are localized on these hippocampal neuron. However, in some neurons, dopamine produced a biphasic response, an outward current was proceeded and an inward current was followed (3 neurons, not shown). In accordance with these results, both D_1 and D_2 receptors were found to be located on the identical hippocampal neuron exerting opposite responses (Fig. 5B).

Some extracellular studies have been reported for the effects of dopamine agonists on hippocampal neurons. Smilowski and Bijak (1987) reported that D_1 agonists decreased and D_2 agonists increased the frequency of spikes from hippocampal neurons. In present study, effects of dopamine agonists on neural activity were in the same direction as theirs, and ionic mechanisms were also elucidated. As in the striatal neurons, significant changes in membrane conductances were not observed but holding currents.

Dopamine Inhibits Nucleus Accumbens Neurons

Nucleus accumbens is the central structure of the limbic system, and it is known to be important for the etiology of schizophrenia (Matthysse, 1981; Defra-

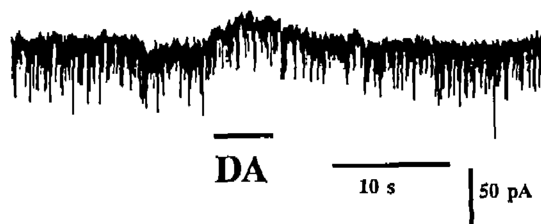


Fig. 6. Effects of dopamine on a nucleus accumbens neuron. A nucleus accumbens neuron cultured for 4 weeks was used. Voltage pulses were omitted in order to precisely observe the effects of agonists on membrane currents. The concentration of dopmine was 10 μM

nce *et al.*, 1985). Nucleus accumbens receives dopaminergic inputs from other brain regions (Anden *et al.*, 1966).

Neurons cultured from the nucleus accumbens, around 18 μm in diameter, were smaller in size than neurons from other brain regions (in case of hippocampus and substantia nigra neurons, around 25 μm in diameter), and it was not easy to make giga-ohm seal for those neurons. Because membrane currents are proportional to the surface area, responses to neurotransmitters were also small in these neurons.

When dopamine was applied to five neurons cultured from the nucleus accumbens, four of them produced an outward current (Fig. 6), suggesting that dopamine inhibited nucleus accumbens neurons. In this study, any specific dopamine agonist was not tried on these neurons. According to Ulchimura *et al.* (1986), this inhibitory response is mediated through D_1 receptors. Using slice preparations they reported that D_1 agonists inhibited nucleus accumbens neurons and cAMP was involved as a second messenger.

Primary Brain Cell Culturing Techniques and Effects of Dopamine Agonists on Various Brain Regions

Cultured brain neurons were useful for studying brain functions. Because of the clean surface of the cultured neurons, it was easy to apply patch clamp technique for electrophysiological studies. Membrane currents were recorded more accurately than conventional microelectrode method. Cultured brain cells were useful for cytological studies also. By virtue of cultured brain cells, immunocytochemical studies were conducted at the subcellular level. Information about neuronal cells, such as morphology, synaptic connections, and distribution of cellular components were provided more in detail.

Different actions of specific dopamine agonists on neurons cultured from different brain regions probably came from the heterogeneity of dopamine receptor subtypes or cellular signaling components. Distinct in molecular level but pharmacologically similar dopamine receptors might have been activated by pharmacologically specific dopamine agonists. Also various signal transduction pathways could be involved in the modulation of brain neurons through dopamine receptors, depending on the origin of neurons cultured from.

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