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## Morphological and Physiological Properties of Serotonergic Neurons in Dissociated Cultures from the Postnatal Rat Dorsal Raphe Nucleus

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### 1. Introduction

Serotonergic neurons in the brain play important roles for the brain functions. These neurons are indispensable for vital functions such as sleep, appetite, nociception, and aggression. Dysfunction of the serotonergic system causes a variety of brain disorders, such as anxiety and depression (Brunton et al., 2006).

Distribution of the serotonergic neurons in the brain was first investigated histochemically with the histofluorescence method (Dahlström and Fuxe, 1964). This was followed by a more precise technique of serotonin immunocytochemistry (Steinbusch et al., 1978; Steinbusch, 1981). Serotonergic neurons are distributed in various raphe nuclei in the mesencephalon, pons, and medulla oblongata. Among these raphe nuclei, the dorsal raphe (DR) nucleus is the largest, containing about a half of the total brain serotonergic neurons (Dahlström and Fuxe, 1965; Descarries et al., 1982; Piñeyro and Blier, 1999). The DR nucleus is the main source of ascending serotonergic pathways which innervate a variety of brain areas such as the cortex, basal ganglia, and amygdala (Azmitia and Segal, 1978; Steinbusch et al., 1981).

We have developed a dissociated cell culture of DR serotonergic neurons from postnatal rats. For this purpose, we used our method of culturing specific brain nuclei. The feature of our method is first to make brain slices of the region containing the nucleus in question, then to isolate and remove the nucleus under the dissecting microscope. Using this method, we have succeeded in making dissociated cultures of cholinergic neurons from the nucleus basalis (Nakajima et al., 1985), noradrenergic neurons from the locus coeruleus (Masuko et al., 1986), dopaminergic neurons from the substantia nigra, dopaminergic neurons from the ventral tegmental area (VTA) (Masuko et al., 1992; Kim et al., 1995), and more recently histaminergic neurons from the tuberomammillary nucleus (Bajic et al., 2004). We have now applied this method to make cultures of serotonergic neurons from the DR nucleus.

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There are several reports on culturing embryonic brain serotonergic neurons (Yamamoto et al., 1981; Rudge et al., 1996; Héry et al., 2000; Nishi et al., 2000). Because serotonergic neurons and their nuclei develop during the fetal period (Altman and Bayer, 1981; Lidov and Molliver, 1982; Wallace et al., 1983; Aitken and Törk, 1988; Lautenschlager et al., 2000), and the contour of the DR nucleus is more well-formed in postnatal rats, we decided to make dissociated neuron cultures of the DR nucleus using 9–12 day-old postnatal rats rather than fetal rats.

In this paper, first we describe the method of making dissociated serotonergic neuron cultures from the DR nucleus of postnatal rats. Second, we show data of electrophysiological experiments on those serotonergic neurons. These serotonergic neurons were functional and responded to neurotransmitters such as bombesin and gastrin-releasing peptide. A preliminary account was reported in an abstract (Yasufuku-Takano et al., 2004).

## 2. Materials and methods

### 2.1 Dissociated cell cultures

Cell cultures of the DR nucleus were prepared from postnatal Long-Evans rats (Charles River Laboratories, Inc.). We used essentially the same procedures as reported previously (Masuko et al., 1986; Nakajima and Masuko, 1996, 1999), except that, instead of newborn rats, we used mainly 10 to 12-day-old rats. Under ether anesthesia, the brainstem, from the midbrain to the upper part of the cervical spinal cord, was removed aseptically. Immediately afterwards, the animal was decapitated to ensure euthanasia. The removed brainstems were immersed in an ice-cold oxygenated balanced salt solution consisting of: 130 mM NaCl, 4.5 mM KCl, 2 mM CaCl<sub>2</sub>, 33 mM glucose and 5 mM piperazine-*N'*, *N'*-bis[2-ethanesulfonic acid] (PIPES) buffer (pH 7.4), and were embedded in agar (3.3–3.5%) in the balanced salt solution. The solidified agar block containing the removed brainstems was attached to the bottom of a cutting dish with alpha cyanoacrylate (instant glue), and immersed in the oxygenated ice-cold balanced salt solution. The agar block containing brainstems was then aseptically sectioned with a Vibratome (Lancer 1000) into 400 μm thick slices.

DR is a long nucleus, rostrally located at the level of the caudal end of the oculomotor nucleus complex, and caudally up to the level of the rostral end of the fourth ventricle (Steinbusch et al., 1981; Törk, 1985). When the brains of 9 to 12-day-old rats were serially sectioned at the thickness of 400 μm, the DR nucleus was contained within three slices. The DR nucleus was identified under the dissecting microscope, and excised from the slices using a pair of microknives that consisted of a 30G needle attached to a tuberculin syringe. We excised dorsomedial, ventromedial, and lateral regions of the rostral mesencephalic part of the DR nucleus. The excised pieces of DR nuclei were treated with oxygenated Leibovitz's L-15 culture medium (GIBCO BRL) containing 12 units/ml papain (Worthington Biochemical Corp.), 1.27 mM DL-cysteine, and 0.2 mg/ml bovine serum albumin (pH 7.3) for 30 minutes at 37°C. In order to remove the papain, the specimens were then rinsed three times with the culture medium, and dissociated by repeated gentle pipetting (Leifer et al., 1984). Dissociated neurons were then plated on a well (12 mm in diameter) made inside a culture dish (3.5 cm in diameter). The floor of the well was pre-coated with rat tail collagen and a glial feeder layer (Nakajima and Masuko, 1996).

The cultures were kept at 37° C at an atmosphere of 10 % CO<sub>2</sub>. The culture medium consisted of minimum essential medium with Earle's salt (88%; GIBCO BRL), modified by adding L-glutamine (0.292 mg/ml, final concentration), NaHCO<sub>3</sub> (3.7 mg/ml), D-glucose (5 mg/ml), and was supplemented with L-ascorbic acid (10 μg/ml), penicillin (50 units/ml), streptomycin (50 μg/ml), and heat-inactivated rat serum (2%, prepared in our laboratory). The cultures were maintained for 10 to 14 days.

## 2.2 Immunocytochemistry

Serotonergic neurons in DR cultures were identified by serotonin immunocytochemistry (Steinbusch et al., 1978; 1981) by using the peroxide-antiperoxidase method (Sternberger, 1979) or by using the indirect fluorescence immunocytochemical method.

**Fixation**—DR neuron cultures were rinsed with cold (4 °C) 0.1 M PBS (pH 7.4) for three times and were fixed first with cold 4 % paraformaldehyde in 0.1 M PBS for 13 minutes, followed by cold 4 % paraformaldehyde in 0.1 M borate buffer (pH 11.0) for 13 minutes.

**Immunocytochemical treatment with the peroxide-antiperoxidase method of Sternberger**—Following the fixation, cultures were rinsed with 0.1 M PBS containing 0.3% Triton X-100, and were incubated in a blocking solution (5% normal goat serum and 0.3% Triton X-100 in 0.1 M PBS) for 15 minutes at 4° C. The specimens were incubated for an hour at 4° C in a primary antibody solution containing rabbit antiserum to serotonin, which was conjugated to bovine serum albumin (Incstar Corp.; 1: 400 in 0.1 M PBS, containing 1% normal goat serum and 0.3% Triton X-100). This process was followed by washing six times with 0.1 M PBS containing 0.3% Triton X-100, and then by one hour incubation in a secondary antibody solution containing fluorescein-labeled goat antibody against rabbit IgG (heavy and light chains: Organon Teknika Corp.; 1:50 in 0.1 M PBS containing 1% normal goat serum and 0.3% Triton X-100) under gentle agitation at room temperature. After three times washing with 0.1 M PBS, the specimens were treated with a rabbit peroxidase-anti-peroxidase complex solution (Sternberger Monoclonals Inc.; 1:80 in 0.1 M PBS containing 1% normal goat serum) at room temperature for an hour. After three times wash with 0.1 M PBS and additional three times wash with 0.05 M Tris buffer (pH 7.4), the specimens were treated with 0.05 M Tris buffer containing 0.02% 3–3'-diaminobenzidine tetrahydrochloride (DAB) and 0.005% H<sub>2</sub>O<sub>2</sub> for up to 10 minutes. After wash with 0.1 M PBS, the specimens were dehydrated, cleared, and cover-slipped using Histomount (National Diagnostics). This processing produced dark brown staining in serotonin-immunoreactive neurons. Serotonin-BSA conjugate (Incstar Corp., Stillwater, MN) was used for absorption experiment to evaluate the specificity of the anti-serotonin antibody. Controls were treated with the same way except for the absence of the primary antibody.

**Immunocytochemistry after electrophysiology**—To determine whether a neuron, which was examined electrophysiologically, was serotonergic, we used the indirect fluorescence immunocytochemical method. The location where this neuron was situated was marked with a circle at the bottom of the culture dish. The culture was fixed and rinsed three times before immunocytochemical treatments. We used rabbit antiserum to serotonin, conjugated with bovine serum albumin, as the primary antibody (Incstar Corp.; 1:400 dilution), and fluorescein conjugated IgG fraction of goat anti-rabbit IgG antibody as the secondary antibody (Organon Teknika Corp.; 1:50 dilution). After incubation with the secondary antibody, the cultures were rinsed with PBS 3 times and mounted on glass slides using fluoromount (Fisher Biotech).

**Immunocytochemistry of neurofilaments using the indirect fluorescence method**—We used mouse monoclonal antibody to neurofilaments (68 KD) (Boehringer Mannheim Biochemicals; 1:3 dilution) as the primary antibody, and rhodamine conjugated goat anti-mouse IgG (whole molecule) antibody (Organon Teknika Corp.; 1:80 dilution) as the secondary antibody. The same procedure as that used for indirect fluorescence immunocytochemistry of serotonin, was used for the neurofilament immunocytochemistry.

## 2.3 Electrophysiology

The whole-cell patch-clamp method was used. During electrophysiological experiments, the culture was continuously superfused with an oxygenated external solution. The 5 mM K<sup>+</sup> external solution, which was used most often, contained: 145.5 mM NaCl, 5 mM KCl, 2.4 mM CaCl<sub>2</sub>, 1.3 mM MgCl<sub>2</sub>, 5 mM HEPES-NaOH, 11 mM D-glucose, and with or without 0.5 μM tetrodotoxin (TTX) (pH 7.4). The 10 mM K<sup>+</sup> external solution was prepared as the above described solution except for the NaCl and KCl concentrations which became 140.5 mM NaCl and 10mM KCl. The intra-pipette solution contained 144 mM K-aspartate, 10 mM NaCl, 5 mM HEPES-KOH, 0.5 mM EGTA-KOH, 0.25 mM CaCl<sub>2</sub>, 3 mM MgCl<sub>2</sub>, 2 mM ATP, 0.1 mM GTP and ~5 mM KOH (pH 7.2). Membrane potential values were corrected for the liquid junction potential between the bath and the patch pipette solutions. 8-OH-DPAT was obtained from Research Biochemicals, Inc; phenylephrine from Sigma Chemical Co, bombesin from Peninsula Laboratories, Inc., and gastrin-releasing peptide was from Peninsula Laboratories, Inc. Unless otherwise indicated, experiments were done at the room temperature.

## 3. Results

### 3.1. Morphological characteristics of cultured serotonergic DR neurons

Dissociated cultures from a brain nucleus are a mixture of different types of neurons and glia cells. We investigated the proportion of serotonergic neurons among other types of neurons and glia cells in our cultures. This was necessary because our purpose was to investigate the brain serotonergic neurons, and a very low proportion of serotonergic neurons would be ineffectual for the experiments.

To determine the proportion of serotonergic neurons in our culture, we must first identify neurons from glia. For this purpose, we performed immunocytochemistry for neurofilaments: neurofilaments serve as a marker of neurons. As an example, in a culture dish, inside a designated area (i.e., a quarter of the total culture area, which was a circle of ~11 mm diameter), the number of cells having processes was 226, and all these cells reacted positively to the antibody against neurofilaments. A small number (totally 11) of neurons that were devoid of processes were also positive to the neurofilament antibody. Flat background cells did not show positive reactivity to antibody to neurofilament: they were probably glial cells of the feeder layer. Because all cells with processes were neurons, and only a small percentage of neurons had no processes, we performed a population statistics on cells with processes only: they would represent all neurons with an error of a few percent.

Table 1 summarizes the survey on cells with processes (namely neurons). The cultures were treated with antibody against serotonin using the peroxidase-antiperoxidase method of Sternberger (1979). Serotonergic neurons, as determined by positive serotonin-immunoreactivity, accounted for 62.4% of the total neurons (cells with processes) examined. Four culture batches were used. We found on average 485 serotonergic neurons in one culture dish. Since one rat brain provided five culture dishes, approximately, 2,500 serotonergic neurons were viable after 11 days of culturing originated from a 10-day-old rat brain. Soma diameters were measured on two culture batches. The diameter of the serotonergic and that of the non-serotonergic neurons were essentially the same (Table 1).

Fig 1A shows serotonergic neurons cultured from DR: they were treated with immunocytochemistry against serotonin. The serotonergic neurons could be classified into two major types, polygonal (Fig. 1B and C), triangular (Fig. 1D), and, less frequently, fusiform (Fig. 1E) with thick processes arising from the opposite poles of the soma. However, non-serotonergic neurons turned out to be also of polygonal or triangular shape. It was impossible to identify serotonergic neurons based on their size and shape.

The main conclusion of this section is that our cultures have abundant cells with processes, and almost all of them are neurons. Out of these neurons (with processes) about 60 % would be serotonergic neurons. Because our cultures contain abundant serotonergic neurons, they will be a useful material for investigating the serotonergic neurons.

### 3.2. Correlations between the response to 8-OH-DPAT and serotonin-immunoreactivity

According to Aghajanian and Wang (1978), serotonergic neurons *in vivo* and in slice preparations are inhibited by serotonin which is secreted from their own collaterals that terminate onto the somato-dendritic regions. This auto-inhibition is through 5-HT<sub>1A</sub> receptors located over the somato-dendritic regions (Feldman and Quenzer, 1983; Barnes and Sharp, 1999). We performed experiments to determine whether the hyperpolarizing response of DR neurons caused by 8-OH-DPAT (1  $\mu$ M), a selective agonist to 5-HT<sub>1A</sub> receptors, can be used to identify serotonergic neurons. We recorded the neuronal response to 8-OH-DPAT with the whole-cell patch-clamp method. The same neuron was examined with fluorescence immunocytochemistry using antibody against serotonin.

Fig. 2A shows that a neuron in Fig. 2B responded to 8-OH-DPAT with a conductance increase together with a slow outward current at the holding potential ( $-77$  mV). These responses under voltage-clamp reflect a hyperpolarization with a conductance increase under current-clamp conditions. Immunocytochemistry revealed that this neuron (Fig. 2 A, B, C) was reactive to antibody against serotonin (Fig. 2C), indicating that the neuron is serotonergic. In contrast, the neuron in Fig. 2E neither responded to 8-OH-DPAT (Fig. 2D) nor was reactive to serotonin-antibody (Fig. 2F), indicating that this neuron was not serotonergic. We performed these experiments on 39 neurons (Table 2). Among 26 neurons that responded to 8-OH-DPAT with a conductance increase, 24 neurons (92%) were serotonergic. Among 13 neurons with no response to 8-OH-DPAT, 3 neurons (23%) were serotonergic. These results indicate that the positive response to 8-OH-DPAT is an approximate criterion for identifying serotonergic neurons.

### 3.3. Properties of the 8-OH-DPAT response

We analyzed effects of 8-OH-DPAT, a specific agonist for 5HT<sub>1A</sub> receptor, on serotonergic neurons. We examined current-voltage (*I-V*) relationships in 5 mM K<sup>+</sup> and 10 mM K<sup>+</sup> external solutions (Fig. 3A). In Fig. 3A, five neurons were examined for each external solution. The 8-OH-DPAT (1  $\mu$ M)-induced currents were obtained by subtracting the resting currents from currents after the application of the agonist. In both external solutions (5 mM K<sup>+</sup> and the 10 mM K<sup>+</sup> solutions), the 8-OH-DPAT-induced conductance showed inward rectification. The reversal potential for 5 mM [K<sup>+</sup>]<sub>o</sub> and that for 10 mM [K<sup>+</sup>]<sub>o</sub> were  $-89.1$  and  $-65.9$  mV, respectively. Both of them were near the potassium equilibrium potential,  $E_K$  ( $-87.4$  mV for 5 mM [K<sup>+</sup>]<sub>o</sub> and  $-69.9$  mV for 10 mM [K<sup>+</sup>]<sub>o</sub> at 20°C). These results strongly suggest that 8-OH-DPAT, through the 5-HT<sub>1A</sub> receptor, activated the G-protein-coupled inward rectifier K<sup>+</sup> (GIRK; Kir3) channel in DR serotonergic neurons.

Barium ions are known to inhibit inward rectifier K<sup>+</sup> channels (Hagiwara et al., 1978). We examined the effect of Ba<sup>2+</sup> on the ability of 8-OH-DPAT to induce the Kir3 (GIRK) channels. As shown in Fig. 3B, we applied a staircase-shaped voltage step (Farkas et al., 1996) to monitor the *I-V* relation. The record in B1 indicates that application of 8-OH-DPAT (1  $\mu$ M) produced a large increase in the channel conductance. As expected, the currents created by 8-OH-DPAT (namely, the difference between the currents before and after the 8-OH-DPAT application) revealed *I-V* relation of an inward rectifying shape, similar to the one in Fig. 3A. We quantified the 8-OH-DPAT effect by using the current amplitude produced by the first step of the staircase voltage imposed (corresponding to a 65 mV hyperpolarization from the holding potential of  $-76$  mV). Subtraction of the current before the 8-OH-DPAT application from that after the 8-



OH-DPAT application gave the current increment that was generated by the 8-OH-DPAT application (the average current increment was  $297 \pm 34$  %; mean  $\pm$  sem,  $n = 5$ ). Next, we examined the effects of barium ions. In the presence of  $\text{BaCl}_2$  (300  $\mu\text{M}$ ), 8-OH-DPAT hardly activated the K current (Fig. 3B2): the current increment induced by 8-OH-DPAT became only  $11 \pm 6$  % (mean  $\pm$  sem,  $n = 5$ ). This means that the  $\text{Ba}^{2+}$  treatment reduced the 8-OH-DPAT effect to the level of 4 % (11/297). As shown in B3, the effects of barium ions were partially reversed by washing the barium ions.

The results of this section strongly suggest that the 8-OH-DPAT-induced inhibition of DR serotonergic neurons is largely due to the activation of a hyperpolarizing current; this current shows inward rectification and is eliminated by barium ions. This mode of 8-OH-DPAT effect probably applies to the natural condition, in which serotonin produces inhibitory effects through the  $5\text{-HT}_1$  receptor. These results suggest that 8-OH-DPAT, through the  $5\text{-HT}_{1A}$  receptor, activated the G-protein-coupled inward rectifier  $\text{K}^+$  (GIRK; Kir3) channel in DR serotonergic neurons.

### 3.4. Action potentials

The properties of action potentials recorded from cultured DR neurons were investigated using current clamp recordings at  $33^\circ\text{C}$ . Experiments were performed on 15 neurons that were likely to be serotonergic since all of them responded to 8-OH-DPAT. Two neurons out of 15 neurons produced spontaneous repetitive firing, whereas the remaining 13 neurons were silent, but produced action potentials when a depolarizing current was applied. Table 3 shows action potential property of these 15 neurons.

### 3.5. Transmitter and agonist effects on action potential firing

We have investigated the effects of various transmitters and agonists on the initiation of action potentials. We chose neurons that responded with a hyperpolarization to 8-OH-DPAT application since these neurons were likely to be serotonergic neurons. Application of phenylephrine (a selective  $\alpha 1$ -adrenoreceptor agonist, 50  $\mu\text{M}$ ), bombesin (1  $\mu\text{M}$ ), or gastrin-releasing peptide (1  $\mu\text{M}$ ) depolarized these neurons and produced action potentials (Fig. 5, A, B, C). The effects of these transmitters and agonists are summarized in Table 4.

Fig. 5D shows that application of 8-OH-DPAT induced a hyperpolarization and the concomitant inhibition of action potentials.

## 4. Discussion

### 4.1. Features of the dissociated DR cultures

One of the features of our dissociated DR cultures is that they originated from  $\sim 10$ -day-old postnatal rats. This is in contrast to the DR cultures that are obtained from embryonic brains (Yamamoto et al., 1981; Rudge et al., 1996; Héry et al., 2000; Nishi et al., 2000) or from newborn rats (Johnson, 1994). This difference could be important for some types of research such as experiments involving neurotransmitter receptors since certain neuroreceptors are known to increase in number postnatally or to develop postnatally (Dudai et al., 1980; Gonzalez et al., 1989). Another advantage of using older postnatal animals is that the DR nucleus is more developed and better defined, allowing us to dissect out the DR nucleus more precisely.

Highly enriched serotonergic neuron cultures are desirable for various types of experiments. Our cultures are rich in serotonergic neurons: 62% of our cultured DR neurons were serotonergic (Table 1). Cultures containing a high percentage of serotonergic neurons were made by our method, which uses selective excision of the DR nucleus from brain slices under the dissecting microscope.

## 4.2. Electrophysiological properties of cultured DR serotonergic neurons

Most of serotonergic neurons in our DR cultures did not show spontaneous firing of action potentials. Similarly, DR serotonergic neurons in culture (Johnson, 1994) as well as those in slice preparations are quiescent (Vandermaelen and Aghajanian, 1983; Kirby et al., 2003). In contrast, many DR serotonergic neurons *in vivo* are firing spontaneously (Aghajanian and Wang, 1978). Absence of spontaneous firing of serotonergic DR neurons in culture and in slice preparations could be due to the absence of excitatory inputs to DR (Baraban and Aghajanian, 1980; Vandermaelen and Aghajanian, 1983; Yoshimura et al., 1985). The duration of action potential (1.7 msec) and the amplitude of after-hyperpolarization (20 mV) obtained from our cultured serotonergic neurons (Table 3) are very similar to those reported for slice preparations (Aghajanian and Lakoski, 1984).

## 4.3. Activation of 5HT<sub>1A</sub> receptor by serotonin

Serotonergic DR neurons *in vivo* or in slices are inhibited (hyperpolarized) by serotonin through their autoreceptors (5-HT<sub>1A</sub> receptors) (Aghajanian and Wang, 1978; Vandermaelen and Aghajanian 1983; Kirby et al. 2003). Aghajanian and Lakoski (1984) noticed that this inhibition is caused by the activation of K<sup>+</sup> channels, which were later identified as G protein-coupled inward rectifier channels (Penington et al., 1993; Jin and Akaike, 1998).

Our cultured serotonergic neurons responded to 8-OH-DPAT (a 5-HT<sub>1A</sub> receptor agonist) with an increase in an inwardly rectifying K<sup>+</sup> conductance (Figs. 2 and 3). The neurons responding to 8-OH-DPAT are mostly (92%: 24 out of 27 cells) serotonergic neurons (Table 2, Fig. 2), indicating a very good, though not perfect, correlation between positive 8-OH-DPAT responses and positive serotonin immunocytochemistry (Table 2). A small percentage of non serotonergic neurons responded to the application of 8-OH-DPAT (17%: 2 out of 12 cells). These results agree with the data on brain slice preparations (Kirby et al., 2003), in which a similar correlation existed between the presence of 5HT<sub>1A</sub> receptors and the presence of serotonin in the neuron.

## 4.4. Responses to excitatory transmitters and agonists

**Phenylephrine (a selective  $\alpha$ 1-adrenoreceptor agonist)**—Noradrenergic afferent fibers innervate DR neurons. Stimulation of these nerve fibers causes tonic excitation in DR serotonergic neurons. This noradrenergic effect is most likely transmitted through the  $\alpha$ 1-adrenoceptors (Baraban and Aghajanian, 1980; Vandermaelen and Aghajanian, 1983; Yoshimura et al., 1985). Activation of  $\alpha$ 1-adrenoceptors inhibits K<sup>+</sup> conductance. This produces depolarization, resulting in an increase in the firing rate (Aghajanian, 1985).

Most of serotonergic DR neurons in cell cultures as well as in brain slices do not generate spontaneous action potentials (Vandermaelen and Aghajanian 1983; Johnson, 1994; Kirby et al., 2003). These results are probably due to the scarcity of excitatory inputs (including noradrenergic inputs) to the DR neurons in these preparations. In slice preparations of DR serotonergic neurons, application of  $\alpha$ 1-adrenoceptor agonist restores firing of action potentials (Vandermaelen and Aghajanian 1983; Kirby et al. 2003).

In our cultures, phenylephrine, an  $\alpha$ 1-adrenoreceptor agonist, produced depolarization and elicited action potentials in all putative serotonergic neurons examined (6 out of 6 cells). In this respect, our primary DR neuron cultures from ~10 day-old postnatal rats behave like the neurons in brain slice preparation, exhibiting the capability of producing action potentials by phenylephrine application.

**Bombesin and gastrin-releasing peptide**—In mammalian tissues, there are two kinds of bombesin-like peptides, gastrin-releasing peptide and neuromedin B. These peptides mediate a variety of biological activities, including the control of body temperature, satiety,

and the maintenance of circadian rhythms (Battey and Wada, 1991). Three types of receptors for bombesin-like peptides were cloned: the gastrin-releasing peptide receptor, the neuromedin B receptor, and the bombesin receptor subtype 3 (Spindel et al., 1990; Battey et al., 1991; Corjay et al., 1991; Wada et al., 1991; Fathi et al., 1993). These three types of receptors exist in the DR nucleus of rats (Ladenheim et al., 1992; Battey and Wada, 1991; Jennings et al., 2003).

Our cultured serotonergic neurons responded to the application of bombesin and gastrin-releasing peptide with depolarization and action potential firing (Table 4; Fig. 5, B, C). These data approximately agree with those obtained using slice preparations by Pinnock et al. (1994).

#### 4.5. Characteristics and future applications of cultured DR serotonergic neurons

We have developed dissociated cultures of the DR nucleus from ~10-day-old postnatal rats. The cultures were rich (~60 %) in serotonergic neurons. Serotonergic and non-serotonergic neurons could not be distinguished by their light microscope morphology. However, we found that neurons responding to the 5-HT<sub>1A</sub> receptor agonist 8-OH-DPAT with hyperpolarization were most likely (~90%) serotonergic. The application of transmitters such as phenylephrine, bombesin, and gastrin-releasing peptide to these serotonergic neurons resulted in neuronal excitation. Such serotonergic neurons also produced action potentials similar to those reported in brain slice preparations. These results indicate that our cultured DR serotonergic neurons are useful for physiological studies. By using our dissociated cultures of brain nuclei, such as the locus coeruleus (Masuko et al., 1986), the substantia nigra (Masuko et al., 1992), and the nucleus basalis (Nakajima et al., 1985), we investigated signal transduction mechanisms of neuronal excitation and inhibition using whole-cell and single channel recordings (Grigg et al., 1996; Hoang et al., 2004). We also performed application of toxins such as pertussis toxin for a long time (Hoang et al., 2003), did injection of antisense oligonucleotides (Takano et al., 1997), and transfected DNAs to cultured neurons with a microinjector (Koike-Tani et al., 2005). Recently, using viral vectors, delivery of DNAs and small interference RNAs to primary cultured neurons have become possible. Application of a variety of methods to our DR nucleus cultures would answer many questions in physiology and pathology of the DR serotonergic neurons.

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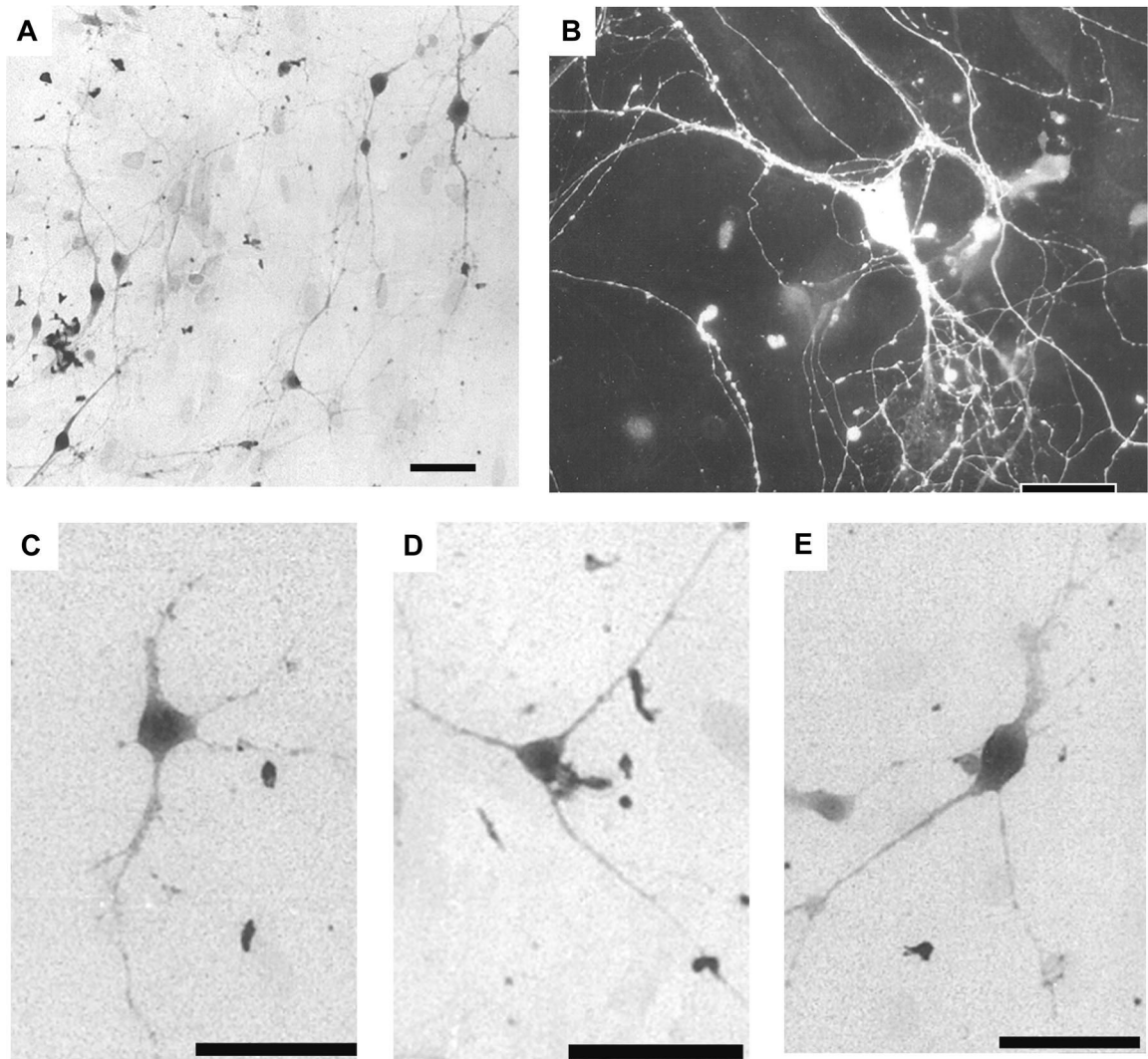
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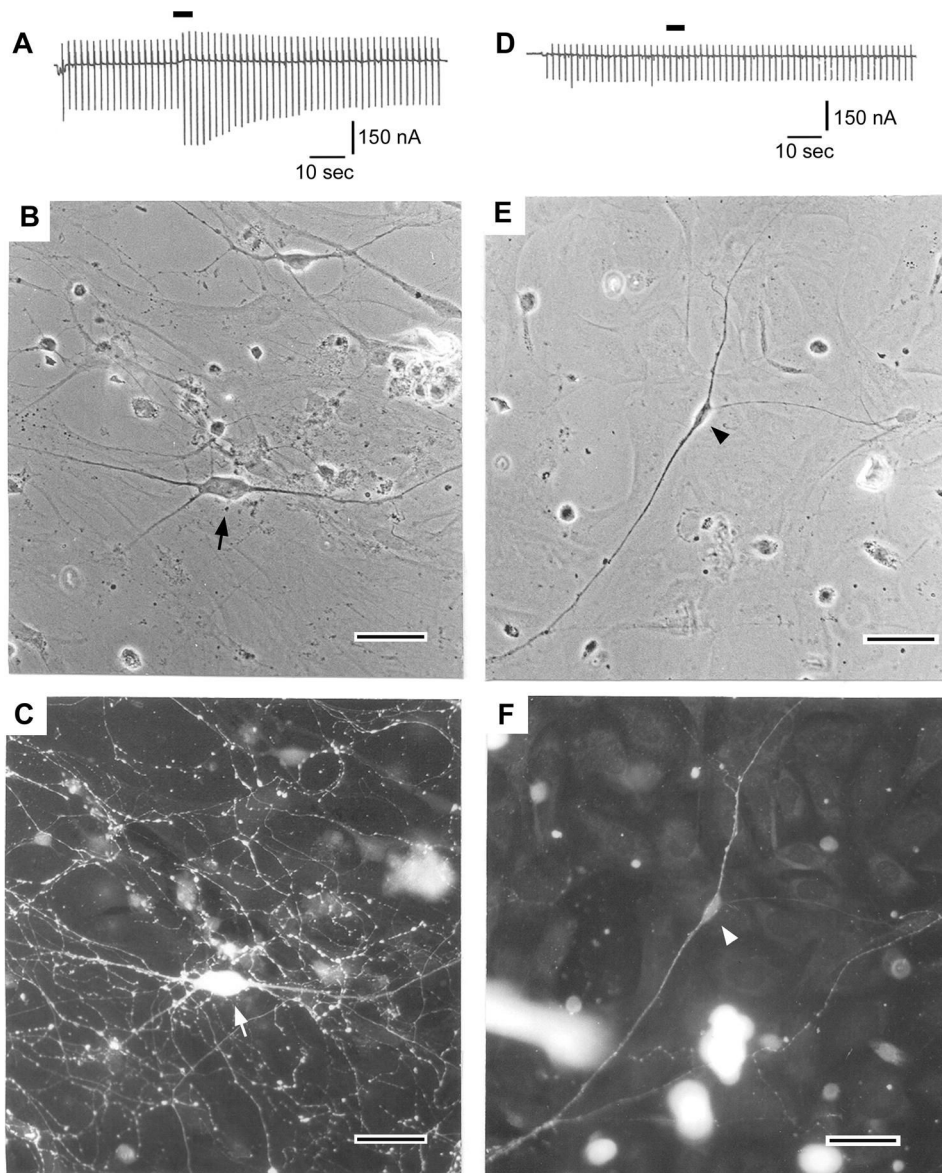
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**Fig. 1. Serotonergic neurons in dissociated cultures from DR**

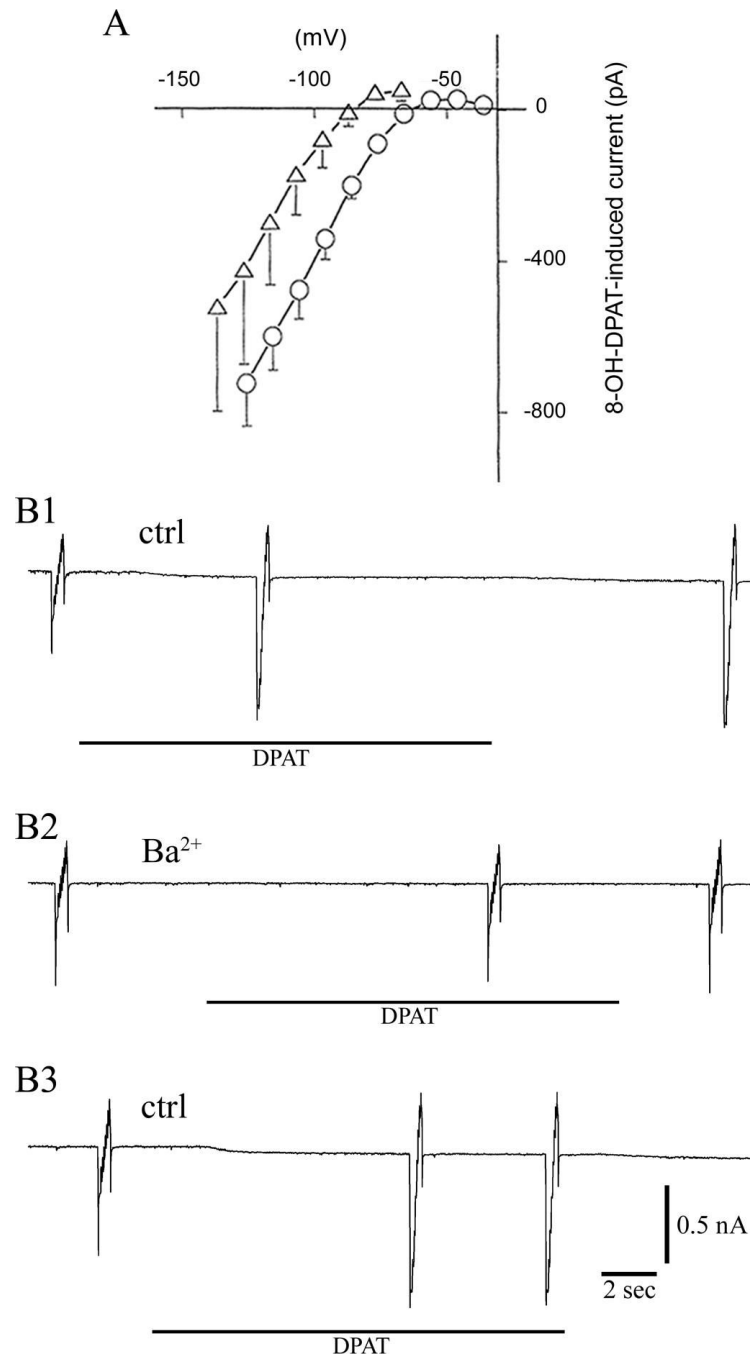
Cells with positive immunoreactivity to serotonin are shown. The peroxide-antiperoxidase method (A and C-E) and the indirect immunofluorescence method (B) were used. **A:** Low magnification micrograph, showing many serotonin-immunoreaction positive neurons. **B-E:** Serotonergic neurons. Polygonal neurons (B and C), a triangular neuron (D), and a fusiform neuron (E) are shown. **B:** A serotonin-immunoreactive neuron treated with the indirect immunofluorescence method clearly shows its thick neuronal processes, rich arborizations, and many varicosities. DR neurons from 10-day-old rats were cultured for 11 days (A), 12 days (C-E), or 16 days (B). Bar: 50  $\mu\text{m}$ .





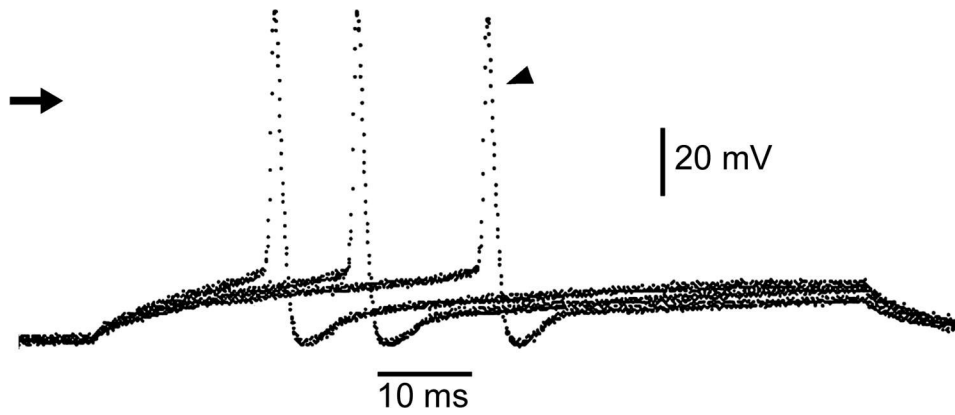
**Fig. 2.** Correlation between the 8-OH-DPAT response and the serotonin immunoreactivity of cultured DR neurons. **(A-C)**: A DR neuron, which responded to 8-OH-DPAT with a conductance increase, was a serotonergic neuron. **(B)**: A phase-contrast micrograph of a neuron (marked by an arrow) after electrophysiological recording but before fixation, from a 7-day culture made from an 11-day-old rat. **(C)**: The same neuron (pointed by arrow) after fixation showing positive reaction to serotonin-immunofluorescence stain. **(D-F)**: An DR neuron, which did not respond to 8-OH-DPAT, was a non-serotonergic neuron. **(E)**: A phase-contrast micrograph of a neuron (marked by an arrow head) after electrophysiological recording but before fixation from 11-day culture made from 12-day-old rat. **(F)**: The same neuron after fixation showing negative reaction to serotonin-immunofluorescence stain. In A and D, conductance changes were monitored by applying a recurrent command pulse sequence consisting of a square-wave depolarization (20 mV, 50 msec) and hyperpolarization (30 mV, 50 msec). The application of 8-OH-DPAT (1  $\mu$ M, 5 sec) was conducted by puff application and marked by horizontal bars. The holding potential was  $-77$  mV and 5 mM  $K^+$  external solution was used.



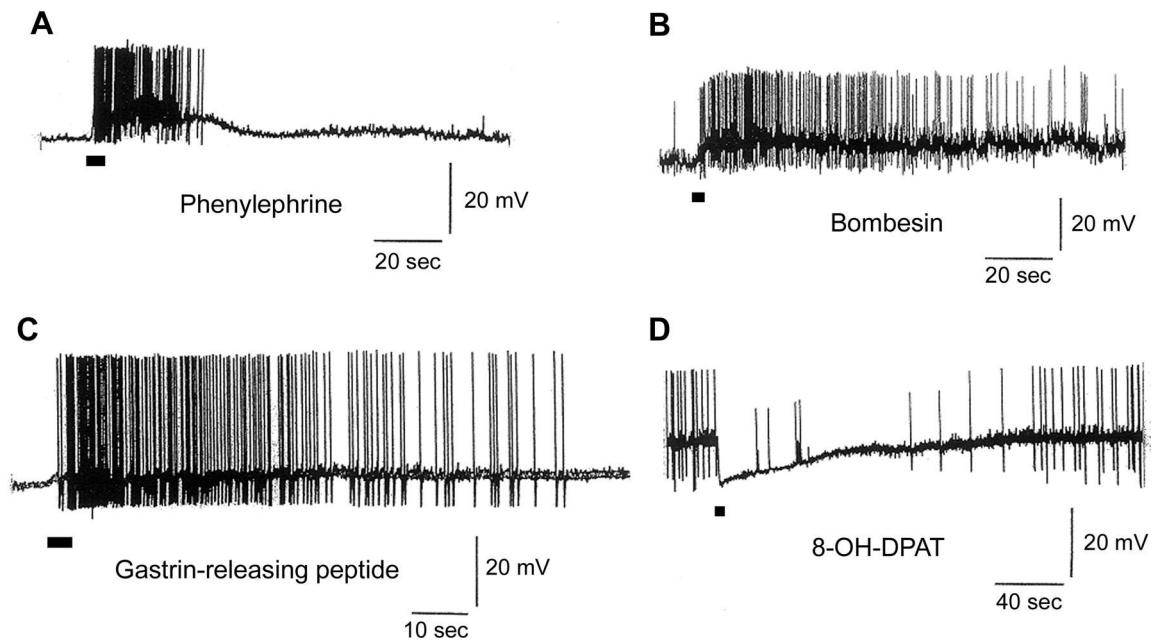


**Fig. 3.** **A.** Current-voltage (*I-V*) relation of 8-OH-DPAT-induced response. The current before the application of 8-OH-DPAT was subtracted from the current after the application of 8-OH-DPAT. In both 5 mM K<sup>+</sup> (triangle) and 10 mM K<sup>+</sup> (circle) external solutions, the conductance showed inward rectification and their reversal potentials were near  $E_K$  (see the text). To measure the *I-V* relation, voltage pulses (50 msec in duration) were applied at a 10 mV increment. Vertical lines indicate S.D. Five neurons were examined for each of different external solutions. The holding potential was  $-77$  mV (5 mM K<sup>+</sup> external solution) and  $-76$  mV (10 mM K<sup>+</sup> external solutions). Neurons cultured for 13 days from 10-day-old rats were used. **B.** Effects of BaCl<sub>2</sub> (300  $\mu$ M) were investigated on 8-OH-DPAT-induced response.

**B1.** The *I-V* relation before and after 8-OH-DPAT (1  $\mu$ M) was measured by imposing staircase-shaped voltage sequence. Application of 8-OH-DPAT produced an inward rectifying current in the same way as in experiments in Fig. 3 A. **B2.** After applying 300  $\mu$ M BaCl<sub>2</sub>, application of 8-OH-DPAT produced hardly any effect. **B3.** After washing out the BaCl<sub>2</sub>, the effect of 8-OH-DPAT was recovered considerably. The interval between the beginning of B1 and that of B2 was 176 sec, while the corresponding interval between B2 and B3 was 190 sec. We have also investigated the effects of BaCl<sub>2</sub> at a lower concentration (100  $\mu$ M): at this BaCl<sub>2</sub> concentration: the increment of current by the application of 8-OH-DPAT was  $22 \pm 16\%$  (mean  $\pm$  sem,  $n = 5$ ) of the control (i.e, no BaCl<sub>2</sub>). In both of these experiments neurons were cultured for 10–19 days from 11-day-old rats.



**Fig. 4.** Action potentials elicited by depolarizing currents (duration, 93 msec). The neuron was cultured for 13 days from a 10-day-old rat. The neuron was probably serotonergic because of the response to 8-OH-DPAT. Arrow indicates the zero current level. The first action potential (marked by an arrowhead) was elicited by the depolarizing current of 150 pA and the second and third action potentials were activated by 170 pA and 190 pA currents, respectively. The resting potential was  $-71$  mV. The whole-cell version of patch-clamp under constant current was used. The experiment was conducted at  $32^{\circ}$  C.

**Fig.5.**

Neurotransmitter and agonist effects on cultured serotonergic DR neurons. All neurons used in this experiment were identified as serotonergic based on their hyperpolarizing response to 8-OH-DPAT. Two different transmitters, bombesin and gastrin-releasing peptide as well as two different agonists, phenylephrine and 8-OH-DPAT, were used. These transmitters or agonists were applied for 5 sec. Phenylephrine, bombesin, and gastrin-releasing peptide depolarized neurons and elicited action potentials (A, B, and C). In contrast, 8-OH-DPAT caused hyperpolarization and inhibited action potential firing (D). Experiments were conducted at 33° C. Cultures were made from 10-day-old rats and maintained for 11 days (D), 13 days (A and C), and 15 days (B).

**Table 1**

Size and occurrence of serotonin-immunoreactive neurons in DR cultures

Immunoreactivity	Diameter ( $\mu\text{m}$ )	Frequency (%)
Serotonin (+)	15.8 $\pm$ 0.3 (n = 250)	62.4 (n = 485)
Serotonin (-)	16.7 $\pm$ 0.5 (n = 108)	37.6 (n = 292)

Cell diameters were determined from two cultures. The frequency measurements were conducted on four cultures. For each batch of cultures, the measurements were conducted on 1/4 of culture area of a dish. All cultured cells were obtained from 10-day-old rats, and were cultured for 11 days. Diameter was defined as a geometrical mean of the major and minor soma diameters. Cultures were treated with the serotonin immunocytochemical method using the peroxide-antiperoxidase method (Sternberger, 1979). Diameters are expressed as means  $\pm$  S.E.M.



**Table 2**  
Serotonin-immunoreactivity (Serotonin-Immun-R) vs. 8-OH-DPAT response in cultured DR neurons

	<b>Serotonin-Immun-R (+)</b>	<b>Serotonin-Immun-R (-)</b>
8-OH-DPAT response (+)	24 neurons (*)	2 neurons
8-OH-DPAT response (-)	3 neurons	10 neurons

The experiments were performed on DR nucleus cultures made from 9 to 12-day-old rats and maintained for 4–16 days. 8-OH-DPAT was applied at 1  $\mu$ M for 5 sec. The indirect fluorescence immunocytochemical method was used.

**Table 3**

## Action potential of cultured serotonergic DR neurons

Soma diameter ( $\mu\text{m}$ )	$19.0 \pm 0.7$
Resting potential (mV)	$-73.0 \pm 2.5$
Action potential height (mV)	$69.2 \pm 2.4$
Action potential duration (ms)	$1.70 \pm 0.07$
Amplitude of after-hyperpolarization (mV)	$20.1 \pm 1.8$

Values are expressed as means  $\pm$  S.E.M. ( $n = 15$ ). Experiments were performed on cultures from 10-day-old rats and maintained for 11–14 days. These 15 neurons were likely to be serotonergic neurons, since they were hyperpolarized by the application of 8-OH-DPAT ( $1 \mu\text{M}$ ). The duration of action potential was measured at threshold. Experiments were conducted at  $33^\circ\text{C}$  using a  $5 \text{ mM K}^+$  external solution.

**Table 4**  
Responses of cultured serotonergic DR neurons to agonists under current clamp

	<b>phenylephrine (50 <math>\mu</math>M)</b>	<b>bombesin (1 <math>\mu</math>M)</b>	<b>gastrin-releasing peptide (1 <math>\mu</math>M)</b>
Action potentials elicited (n)	6	4	6
No response (n)	0	2	1
Hyperpolarized (n)	0	0	0

Agonists were applied for 5 sec. All neurons in this table were likely to be serotonergic, since they responded to 8-OH-DPAT with hyperpolarization. Experiments were conducted at  $\sim 33$  °C, except two neurons in the phenylephrine experiments that were recorded at room temperature. Cultures were prepared from 10 to 12- day-old rats and maintained for 10 to 19 days. 5 mM  $K^+$  external solution was used. n: number of neurons.