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Serotonin Modulates Oscillations of the Membrane Potential in Isolated Spinal Neurons from Lampreys

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Studies were performed on spinal neurons from lampreys isolated by an enzymatic/mechanical method using pronase. The effects of 100 µM serotonin (5-HT) on membrane potential oscillations induced by a variety of excitatory amino acids were studied. 5-HT was found to depolarize branched cells (presumptive motoneurons and interneurons) by 2–6 mV without inducing membrane potential oscillations. However, when oscillations were already present because of an excitatory amino acid, 5-HT changed the parameters of these oscillations, increasing the amplitudes of all types of oscillations, increasing the frequency of irregular oscillations, and increasing the duration of the depolarization plateaus accompanied by action potentials. Serotonin modulation of the effects of excitatory amino acids and the electrical activity of cells in the neural locomotor network facilitates motor activity and leads to increases in the contraction of truncal muscles and more intense movements by the animal. The possible mechanisms of receptor coactivation are discussed, along with increases in action potential frequency and changes in the parameters of the locomotor rhythm.

Serotonin (5-hydroxytryptamine, 5-HT) is a widely recognized modulator of the activity of motor, sensory, autonomic, and several other systems in the vertebrate CNS [46]. Endogenous 5-HT has been found in the CNS of cyclostomata; the homogenized lamprey spinal cord has 5-HT concentrations which are unusually high – 4.88 times higher than in the homogenized spinal cord of the rat [15, 16]. The major sources of 5-HT in the lamprey spinal cord are fibers descending from the midbrain and medulla oblongata, intraspinal interneurons located along the midline of the brain ventral to the central canal, and cells located in the spinal ganglia and along peripheral sensory fibers [23, 41]. The processes of serotoninergic fibers of different origins form dense plexuses both in the ventral and dorsal and in the ventrolateral parts of the spinal segments [23, 40] and thus involve all types of neurons in the locomotor generator, i.e., the spinal center for controlling movement in these animals [20, 21].

These data lead to the suggestion that 5-HT is involved in controlling movement activity. This hypothesis is supported by

experiments on in vitro isolated lamprey spinal cord. Experiments have demonstrated that 5-HT modulates the locomotor rhythm recorded in the ventral roots, this rhythm being induced by addition of glutamate to the per-fusion medium [22]. 5-HT did not disrupt the rhythm of sequences of discharges in the roots on the opposite side of the cord, but did alter the parameters of this rhythm: the frequency of trains of action potentials decreased and their duration increased, and these changes were accompanied by increases in the intersegment phase shift [12, 33].

Thus, 5-HT has been shown to be able to modulate the "motor program" of "fictive swimming" [12, 45].

The locomotor rhythm is based on oscillations in the membrane potential of a number of types of spinal neuron, i.e., presumptive pacemakers [10, 43]. The locomotor rhythm is not generated when only 5-HT is added to the perfusion medium, though it can easily be elicited by addition of low concentrations of excitatory amino acids: NMDA, kainate, glutamate, and aspartate [10, 13, 18]. We have recently shown that excitatory amino acids can induce oscillations in the membrane potential in individual completely isolated cells from the lamprey spinal cord [3, 6]. It was suggested that in the isolated cord, these oscillating cells could synchronize the activity of the locomotor-generating neural network, inducing "fictive swimming." It was also demonstrated that 5-HT produces different effects on the parameters of excitation and inhibition in isolated spinal neurons of different functional types [5].

Understanding of the mechanism of serotonin-mediated modulation of the "motor program" in cord preparations requires knowledge of whether 5-HT affects membrane potential oscillations evoked by excitatory amino acids in individual rhythm-generating cells. The aim of the present work was to study changes in membrane potential oscillations parameters induced by 5-HT in multipolar (branched) spinal neurons exposed to excitatory amino acids.

Preliminary results from these studies have previously been published in brief [11].

Methods

Experiments were performed on isolated neurons from the spinal cords of adult lampreys of the species *Lampetra fluviatilis* using a whole-cell patch-clamp technique. The methods for isolating spinal cord, enzymatic/mechanical treatment of slices, constructing the chamber, and the techniques used for making contact between the cell cytoplasm and intrapipette solutions have all been described previously [1, 2, 4]. The soft meninges were removed and cord tissue was dissociated using collagenase (1.6 mg/ml) and pronase (0.5 mg/ml). Branched cells with 2–5 processes of length up to 300 µM were collected in cooled aerated physiological saline by careful pipetting using Pasteur pipettes with flamed tips with diameters decreasing from 1000 to 200 µM. The physiological saline contained 92.0 mM NaCl, 2.5 mM KCl, 2.6 mM $CaCl_2$, 2.4 mM $MgCl_2$, 20.0 mM HEPES-Na, 0.3 mM EGTA, 3.0 mM $NaHCO_3$, 0.75 mM NaH_2PO_4, 0.25 mM $Na2HPO_4$, and 10.0 mM glucose, pH 7.4.

The state of each cell selected for experiments was initially assessed using a number of measures: the initial membrane potential, which ranged from −30 to −60 mV, the presence of action potentials with amplitudes of 70–100 mV, which could be elicited in current-clamping conditions, and the presence of Na^+/K^+ currents with amplitudes of 20–40 nA on stepping the potential towards depolarization from an initial level of −80 (or −100) mV. Cells selected according to these criteria were fixed on the patch-clamping pipette and placed in a 0.5-ml chamber, which was continuously perfused with oxygen-aerated physiological saline cooled to 6–10°C; during experiments, this was supplemented with excitatory amino acids and 5-HT. The following excitatory amino acids were used: glutamate (1 mM), aspartate (1 mM), kainate (0.5 mM), NMDA (1 mM, mixed with 2 µM glycine), and 5-HT (100 µM). All reagents were obtained from Sigma (USA). Patch-clamping pipettes were made from microhematocrit capillary tubes and were filled with solution containing 110 mM KF, 20 mM Tris, 5 mM EGTA, and 10 mM glucose, pH 7.2. After verification of the state of the cell, membrane currents were fixed at a level such that the membrane potential was −80 mV; experimental programs were started once the potential had been

stable for 5–7 min. All oscillations in membrane potential were recorded continuously for 1 or 3 min and recorded in a single data file.

The instrumentation included an Axopatch-1D amplifier and a DigiData-1200 (Axon Instruments, USA) analog-to-digital converter. Experimental data were collected and analyzed using four programs of the pCLAMP 6.0 suite (Axon Instruments, USA). Responses were recorded on the hard disk of a Pentium 2 computer. Data were presented graphically using the program SigmaPlot.

Results

A total of 25 branched neurons were used for studies of membrane potential oscillations during perfusion with solution containing N-methyl-D-aspartate (NMDA) in glycine. In 21 of these cells, application of 100 μM 5-HT affected the parameters of evoked oscillations. Typical examples were given in Figs. 1 and 2.

As we have already noted in previous reports, oscillations can be irregular or regular (rhythmic). Irregular oscillations can be dominated by rare long-lasting depolarization jumps of essentially uniform amplitude; this can be regarded as a sign of a bistable membrane potential (Fig. 1, *A*, *3*, *4*). Another type of irregular oscillation consists of transient frequent oscillations of low amplitude (Fig. 1, *B*, *7*, *8*).

It was demonstrated that 5-HT can modulate all types of oscillations. As shown in Fig. 1, perfusion with physiological saline without addition of 5-HT had the effect that the membrane potential of cell "A" was stable at a level of −76 mV (during 5 min of continuous observations). Stimulation with transient depolarization impulses evoked action potentials, after which no further cell activity was seen (Fig. 1, *A*, *2*). Addition of NMDA with glycine to the per-fusing solution evoked depolarization (−40 mV, 8 sec), after which the membrane potential returned to initial, but was unstable (Fig. 1, *A*, *3*, *4*). Traces showed jumps of depolarization with amplitudes of 16–32 mV and durations ranging from 200 msec to 2–3 sec. After recording oscillations of this type for 17 min, the NMDA/glycine solution was supplemented with 100 μM 5-HT; the nature of cell activity changed

within 1 min of perfusion with this solution – the amplitude and duration of jumps increased and a depolarization plateau appeared, this lasting up to 6–7 sec (Fig. 1, *A*, *5*, *6*). After perfusion with this solution for 10–20 min, cell activity generally started to quieten and then ceased. Despite the fact that the oscillations of cell "B" were different in nature (Fig. 1, *B*, *7*, *8*), the effects of 5-HT on its oscillations were fundamentally the same – the frequency and amplitude of transient jumps increased, the depolarization plateau increased in amplitude to the action potential generation threshold, and the duration increased to 5.2 sec (Fig. 1, *B*, *9*, *10*). Activity quietened after 10–20 min.

Stable increases in the frequency of irregular oscillations in response to 5-HT were seen in all the cells studied. This effect was assessed quantitatively by plotting diagrams of the numbers of oscillations per min in different perfusion solutions. Figure 2 shows the results obtained from three neurons (*A*, *B*, *C*). The vertical axis of each of the three plots (*A*, *B*, *C*) shows the number of oscillations (*n*) with amplitudes of less than 10 mV over 1 min of perfusion with the solution indicated in the upper part of each plot. Numbers (minutes) on the horizontal axes show the duration of preliminary perfusion with the solution indicated, after which counts of the numbers of oscillations were made. As shown in Fig. 2, regardless of whether oscillations were seen in physiological saline (*A*, control) or not (*B*, control), addition of NMDA with glycine to the solution induced activity in all cells, and addition of 5-HT to this solution induced multiple and long-lasting (up to 14 min) increases in the frequency of oscillations, which subsequently gradually disappeared.

5-HT could be seen to have similar effects in cases in which membrane potential oscillations were induced by other amino acids, such as aspartate (*n* = 4). Aspartate induced waves of oscillations (Fig. 3, *A*, *2*) in an initially "quiet" neuron (Fig. 3, *A*, *1*). After addition of 100 μM 5-HT to the solution, the waves of depolarization became more regular and their amplitude increased (Fig. 3, *A*, *3*), continued in this way for 10–12 min, and then showed decreases in activity despite the continuing presence of aspartate and 5-HT in the solution.

Glutamate, as we have previously demonstrated [6], generally induced short, high-amplitude oscillations, sometimes in "trains." These oscillations, recorded in seven cells, were also potentiated by serotonin (Fig. 3, *B*, *3*). "Glutamate" oscillations could reach frequencies of up to 50–70 oscillations per minute in the presence of 5-HT, this persisting for 10–15 min in the presence of glutamate and after 15–20 min of washing.

Another characteristic of the actions of 5-HT on the cell membrane potential and currents was also noted during perfusion with excitatory amino acids. In many cells with stable initial membrane potentials and normal action potentials evoked by a short stimulus (Fig. 4, *A*), perfusion with physiological saline containing NMDA and glycine for 5–8 min led to the appearance of irregular oscillations which included long-lasting depolarization plateaus reaching the threshold amplitude for generating action potentials (Fig. 4, *B*). The constant nature of depolarization sometimes persisted for several seconds. After addition of 5-HT to the per-fusing solution (Fig. 4, *C*), the durations of these depolarization plateaus increased and could reach 8–10 sec. It is possible that this type of long-lasting change in the membrane potential may provide sigifnicant corrections to cell activity, though its importance ultimately remains unclear. Plateaus with lower levels of depolarization are more understandable; these were often accompanied by action potentials (Fig. 4, *D*).

As noted above, 11 of 25 cells with electrical parameters within normal limits did not respond to perfusion with solutions containing excitatory amino acids by producing oscillations in the membrane potential. We also tested the stimulation of oscillations in these cells by addition of 5-HT to the perfusing solution. In all cases, the results obtained were not particularly persuasive. Traces from one of these experiments are shown in Fig. 5. This shows that NMDA-induced oscillations were not marked, while addition of 5-HT to the solution, although making the oscillations more regular, had little effect on the amplitude or frequency of oscillations, i.e., 5-HT had no obvious effect.

In 23 branched neurons from lamprey spinal cord, we recorded regular (rhythmic) oscillations which appeared in response to addition of 1 mM NMDA (with 2 µM glycine) or 0.5 mM kainate to the washing

solution. Regular oscillations often appeared immediately after a high-amplitude depolarization jump induced by addition of amino acids to the chamber or even as this depolarization decayed. The amplitudes of the regular oscillations in different cells ranged from 2 to 10 mV; frequencies were low, from 0.5 to 1.5 Hz, and corresponded to the frequency of the locomotor rhythm as recorded in the ventral roots in isolated cord preparations [12]. The amplitudes of regular oscillations depended on the membrane potential: oscillations appeared at −60 mV, were of maximal amplitude at −80 mV, and decreased at more negative potentials. The frequency of these oscillations changed only slightly as the membrane potential was altered, and was more dependent on the internal, uncontrolled properties of cells. Regular oscillations, like irregular oscillations, were sensitive to the actions of 5-HT. Treatment with 5-HT was tested in five neurons. The amplitude of regular oscillations increased on addition of 5-HT to the perfusing solution by 52–90% of initial, and recovered after washing (Fig. 6, *A*). The effects of 5-HT on the frequency of regular oscillations were less significant. While the amplitude of oscillations increased by more than 70% in response to 5-HT, the frequency of oscillations decreased, as shown in Fig. 6, *A*. In those cells in which the amplitude of oscillations increased insignificantly in response to 5-HT, there were small increases in the frequency of oscillations (Fig. 6, *B*, *C*), though not extending beyond the normal range of membrane potential oscillations in physiological saline containing excitatory amino acids.

Discussion

The main result of the present studies is the demonstration of the effects of 5-HT on the parameters of regular and irregular oscillations evoked in isolated spinal neurons from lampreys by addition of a variety of excitatory amino acids to the perfusing solution. We will first note that 5-HT alone, at concentrations ranging from 10 μM to 1 mM, induced no activity in isolated cells, this including oscillations in membrane potential. 5-HT also produced no clear effects in cells which did not produce oscillations when treated with excitatory amino acids. These data lead to the suggestion that changes in oscillation parameters seen in the presence of 5-HT, i.e., significant increases in the amplitudes of all types of membrane potential oscillations and increases in the frequency of irregular oscillations,

were evoked by coactivation of serotoninergic receptors and excitatory amino acid receptors.

Similar results have been obtained in other systems. Thus, for example, studies on larvae of the frog *Rana temporaria* showed that addition of 0.1 mM NMDA to the solution perfusing isolated spinal cord, in the presence of tetrodotoxin, only induced depolarization of cells by 20 mV, while further addition of 5-HT in the same solution led to stable oscillations of the membrane potential with amplitudes of up to 40 mV [37]. The authors of these studies noted that there were no oscillations when 5-HT was added to the solution alone or in conditions of blockade of oscillations induced by NMDA in the presence of 5-HT by use of the NMDA receptor antagonist 2-amino-5-phosphovaleric acid [36]. Similar results were also obtained in the spinal cords of *Xenopus* embryos and young larvae. NMDA-induced depolarization (20 mV) did not develop into oscillations of the membrane potential, while addition of 2–5 µM 5-HT to the solution induced stable oscillations in 12% of embryos and 70% of larvae. 5-HT alone did not induce oscillations. The authors concluded that coactivation of 5-HT and NMDA receptors is needed for stimulating the typical locomotor rhythm and generating motor activity [34]. We suggest that coactivation of 5-HT receptors and excitatory amino acid receptors leads to modulation of the processes underlying the generation of oscillations triggered by excitatory amino acid receptors.

There is another way in which 5-HT can influence nerve cells with the ability to show oscillations. Studies of cells from the subglottal, supraglottal, and parietal ganglia of the common snail demonstrated the ability of 5-HT to stimulate neuron activity, eliciting depolarization and initiating the generation of slow waves of membrane potential [7, 8]. In the neonatal rat spinal cord [9] and sympathetic preganglionic neurons in rat spinal cord slices [30], 5-HT also acts as a mediator and can "independently" induce locomotor activity in the form of rhythmic oscillations of the membrane potential in populations of previously "silent" neurons. However, in the lamprey spinal cord, 5-HT is unlikely to show this type of action.

Data have been obtained indicating that increases in the amplitude of oscillations are associated with 5-HT-induced potentiation

of responses to NMDA and AMPA receptors to the application or perfusion of cells with the corresponding excitatory amino acids. The potentiating action of 5-HT on NMDA responses has been demonstrated on neocortical neurons from rats [29, 31, 32] and cats [29]. 5-HT, acting on 5-HT2 receptors, facilitates motor behavior (scratching) induced by NMDA and AMPA in mice [28]. 5-HT is also known to increase depolarization induced by application of glutamate, aspartate, NMDA, and quisqualate in frog motoneurons [24]. Increases in NMDA currents in response to addition of 5-HT to the perfusing solution have been seen in neocortical neurons from rats [32]. Our previous report demonstrated that 5-HT potentates both depolarization and currents evoked by NMDA in lamprey branched cells with the ability to undergo membrane potential oscillations [6].
Another possible cause of increases in the amplitude of membrane potential oscillations is facilitation by serotonin of neurotransmitter release [35, 39]; another is that it may have effects on the operation of intracellular metabolic systems, particularly those associated with activation of adenylate cyclase [14]. However, there are still insufficient data on the molecular mechanisms of serotonin coactivation of responses to application of excitatory amino acids.

While increases in amplitude were characteristic of all types of oscillations recorded, the increases in frequency in response to 5-HT were typical mainly of irregular oscillations (Figs. 1–3). One reason for the increase in frequency of membrane potential oscillations may be the 5-HT-evoked depolarization of branched neurons, usually amounting to 2–6 mV but in some cells reaching 9–25 mV [5]. Increases in the frequency of oscillations at lower membrane potentials (−50 mV) as compared with those at higher potentials (−70 mV) have also been seen in lamprey spinal neurons in isolated cord preparations [42]. As regards regular oscillations, changes in response to 5-HT were not so obvious, and 5-HT could even induce changes in opposite directions. The reasons for this pattern of modulation remain unclear.

Increases in the amplitude and duration of depolarization plateaus can be regarded as the characteristic feature of the action of 5-HT on membrane potential oscillations arising in response to excitatory amino acids, especially NMDA and aspartate. These

depolarization plateaus can reach amplitudes of 20–40 mV in solution containing NMDA, and can last up to 6–10 sec (Fig. 5). Since these changes were also present in solutions containing tetrodotoxin [6, 19], it can be suggested that the major ions responsible for maintaining the depolarization plateau are Ca^{2+}, along with the poorly inactivated Ca^{2+} current. Evidence for the involvement of Ca^{2+} in forming the plateau is particularly clearly provided by the fact that substitution of Ca^{2+} with Mn^{2+} prevents the plateau from appearing [25]. Another ion playing an important role in forming the second stable membrane potential level is the K^+ ion, as the Ca^{2+}-dependent K^+ current "disrupts" the plateau and facilitates a return to the initial potential. If the depolarization plateau reaches the threshold for generating action potentials, then numerous discharges arise during the plateau, these being particularly long-lasting in the presence of 5-HT. This pattern is often seen in cat [26] and tortoise [25] spinal cord motoneurons. In isolated lamprey neurons, action potentials appeared less frequently during the depolarization plateau (Fig. 6, *D*). In our experiments, the depolarization plateau evidently was often significantly in excess of the generation threshold for action potentials, which inactivate the currents involved in this process. Data obtained in isolated cells and in cord preparations suggest a possible relationship between the appearance of long-lasting depolarization plateaus accompanied by action potentials and 5-HT-induced changes in the parameters of the locomotor rhythm in cord preparations. Increases in the duration of action potentials in ventral roots can lead to stronger contractions of the truncal muscles and more intense movements by the animals.

Increases in the frequency of potential-activated action potentials arising in response to electrical impulses of a given strength in lamprey spinal interneurons and motoneurons are also seen in conditions of local application of 5-HT alone. Many authors believe that the main reason for the facilitating action of 5-HT on the activity of these cells is a decrease in the afterhyperpolarization of action potentials, which is induced by the 5-HT-medicated suppression of the Ca^{2+}-dependent K^+ current [27, 44, 45]. Increases in the discharge frequency of these neurons can also result in changes in the locomotor activity of the nervous system.

While increasing the duration of action potential discharges in the ventral roots, 5-HT did not disturb the synchronization of the rhythm in the contralateral roots of the same segment. It may act not only on neuron excitation, but it may also prolong the period of neuron "silence," apparently by activating inhibitory interneurons and increasing the release of inhibitory neurotransmitters. This mechanism of action for 5-HT is known for GABA [17, 38], though it has not been studied in lampreys.

The data presented here, along with published data and the considerations laid out above, lead to the conclusion that 5-HT plays an important role in controlling locomotion in lampreys, modulating the activity of both individual ion channels and membrane receptors and the electrical activity of spinal excitatory and inhibitory neurons in the locomotor network. All of these points lead to the conclusion that 5-HT is an endogenous modulator which is actively involved in controlling the motor behavior of cyclostomata.

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Appendix

Figure 1
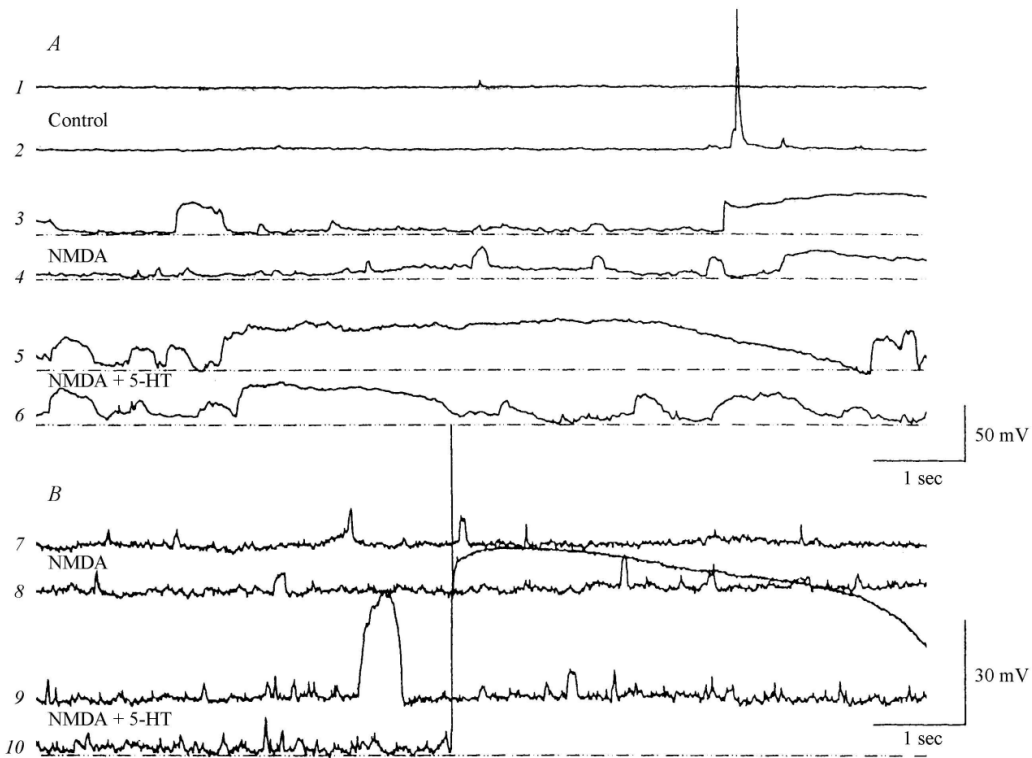
The effects of 5-HT on oscillations evoked by addition of 1 mM NMDA and 2 mM glycine to the solutions perfusing two cells (*A*, *B*). *A*) Membrane potential in normal physiological saline (*1*, *2*), 3 min after addition of NMDA + glycine to the solution (*3*, *4*), and 2 min after addition of 5-HT (*5*, *6*). The constant membrane potential, noted by the dotted line, corresponds to −76 mV. *B*) Oscillations in the membrane potential in another cell after addition of NMDA + glycine to the solution (*7*, *8*) and 5 min after addition of 5-HT to the same solution (*9*, *10*). The constant membrane potential was −95 mV.

Figure 2
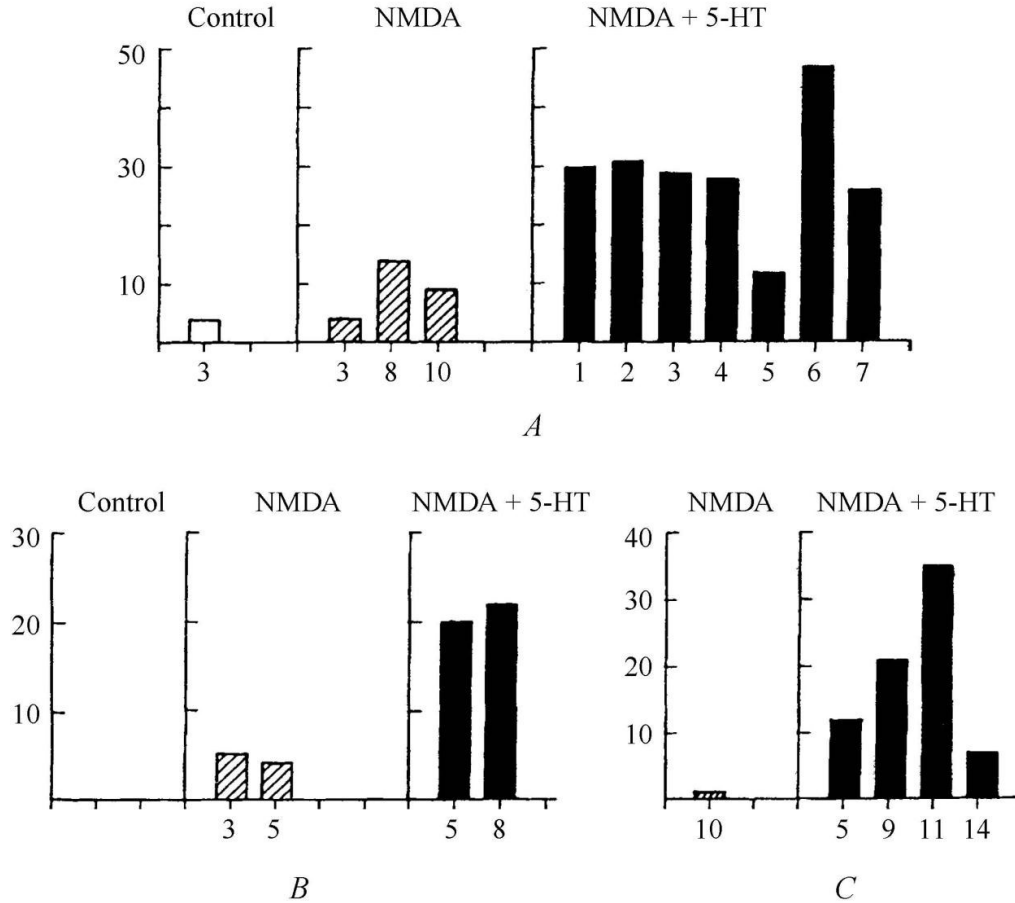
The effects of serotonin on the frequency of oscillations evoked by NMDA + glycine in three branched cells (*A*, *B*, *C*).*n* is the number of oscillations per minute in physiological saline (white columns), in solution containing 1 mM NMDA and2 mM glycine (shaded columns), and in the same solution also containing 100 mM 5-HT (black columns). The horizontal axes show time, min; the vertical axes show *n*.

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Figure 3
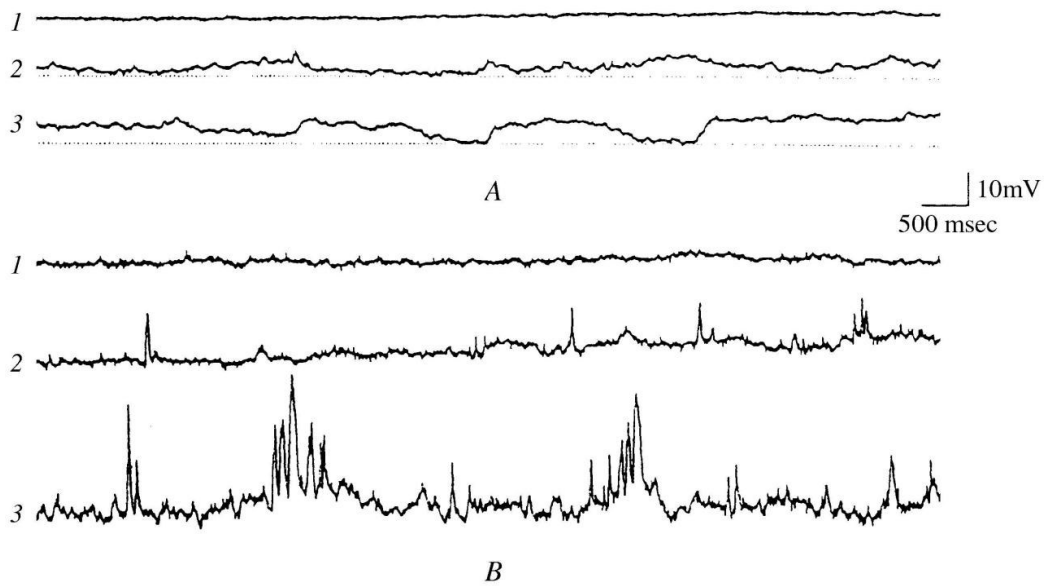
Serotonin modulation of oscillations induced by aspartate and glutamate. *A*) Membrane potential in physiological saline (−100 mV), stable for 10 min (*1*); membrane potential oscillations after exposure to 1 mM aspartate for 6 min (*2*), and after 10 min of perfusion with solution containing aspartate and 100 mM 5-HT (*3*). *B*) Membrane potential in another cell (−100 mV), stable for 5 min of recording (*1*); membrane potential oscillations 5 min after addition of 1 mM glutamate to the solution (*2*); modulation of oscillations 3 min after addition of 5-HT to the solution (*3*). The initial membrane potential was relatively stable in all solutions.

Figure 4
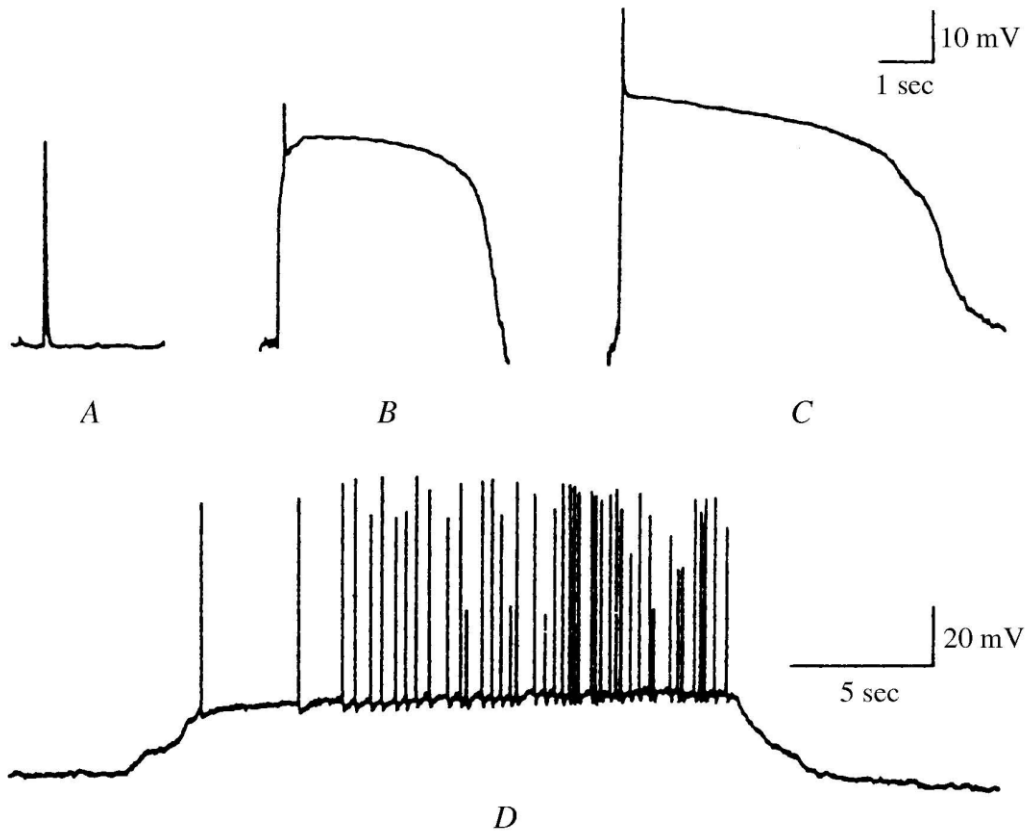
The bistability of membrane potential in solutions containing NMDA and 5-HT. Action potential evoked by short stimuli in normal physiological saline (*A*); spontaneous jumps in the potential to a new level after perfusion of the cell for 8 min with NMDA + glycine (*B*); as *B*, after 5 min of perfusion with solution containing 5-HT (*C*). All traces were made on a single cell. The constant membrane potential was −80 mV. *D*) A depolarization plateau in another cell perfused with solution containing NMDA, glycine, and 5-HT. The constant membrane potential was −60 mV.

Figure 5
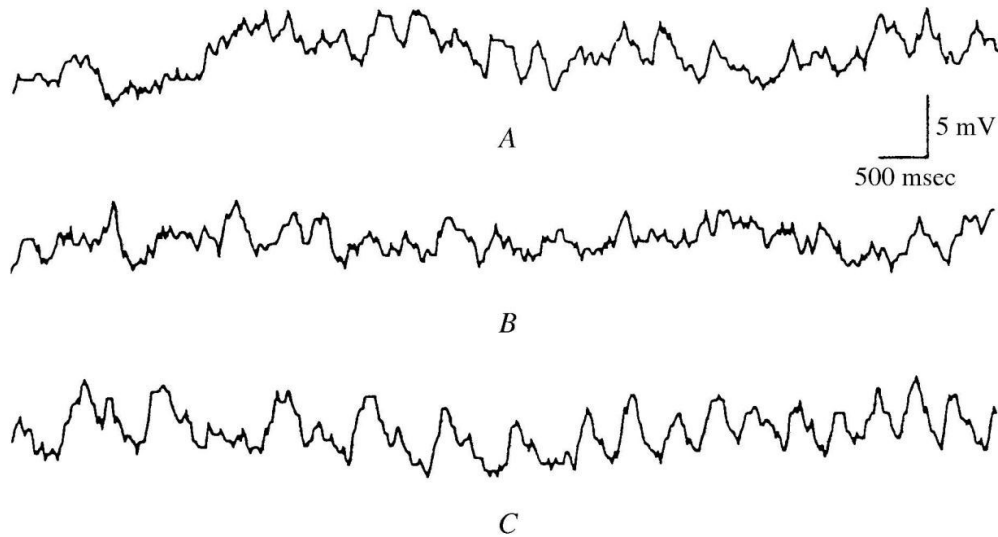
Membrane potential oscillations in a branched cell exposed to NMDA + glycine and 5-HT. Membrane potential oscillations in normal physiological saline (*A*); after 10 min of perfusion in solution containing NMDA + glycine (*B*) and after 7 min of perfusion in the same solution supplemented with 5-HT (*C*). The initial membrane potential was −80 mV. All traces were made from a single cell.

Figure 6
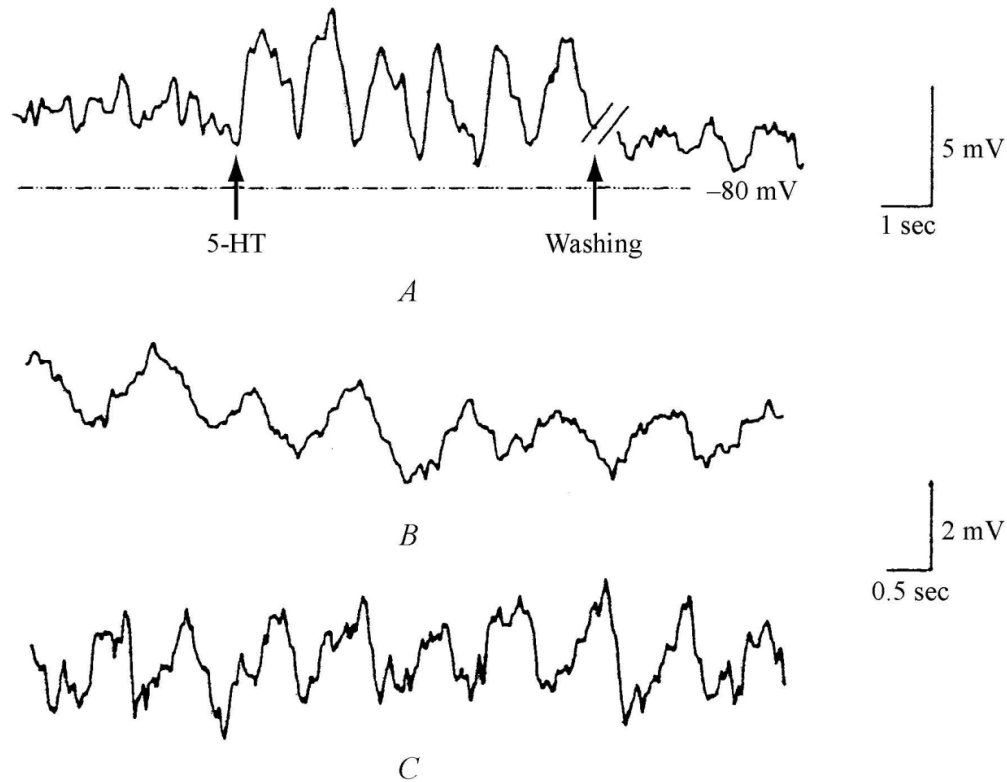
The effects of 5-HT on regular oscillations evoked in two branched cells by addition of kainate to the perfusing solution. *A*) Oscillations in solution containing kainate after addition of 100 mM 5-HT to the chamber (the moment of addition is identified by the arrow) and after 5 min of washing (the washing time is identified by two diagonal lines). The amplitude of oscillations in solution containing 5-HT increased by 87.5%, while the frequency decreased by 50%. *B*, *C*) Oscillations in another cell after 5 min of perfusion with 0.5 mM kainate (*B*) and after 2 min of perfusion with solution containing kainate and 100 mM 5-HT (*C*). The constant membrane potential was −90 mV. The amplitude of oscillations in solution containing kainate increased by 66%, while the frequency increased by 37.1% of initial.