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Ethanol Increases the Firing Rate of Dopamine Neurons of the Rat Ventral Tegmental Area *In Vitro*

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Abstract

The ventral tegmental area (VTA) is a brain region rich in dopamine-containing neurons. Since most agents which act as substrates for self-administration increase dopaminergic outflow in the mesolimbic or mesocortical areas, the VTA slice preparation may be useful for identifying drugs with potential for abuse. While ethanol (EtOH) is a drug of abuse which has been widely studied, the properties of ethanol which contribute to its abuse potential are not known. We have developed a brain slice preparation of the VTA in order to study the action of EtOH on putative dopamine neurons. Concentrations of EtOH from 20 to 320 mM produce a dose-dependent excitation of the dopamine-type neurons of the VTA. About 89% of neurons which have electrophysiological characteristics established for presumed dopamine-containing neurons were excited by ethanol in the pharmacologically relevant concentration range. This excitation persists in low calcium, high magnesium medium, which suggests a direct excitatory action of EtOH on dopamine-type cells in the VTA slice.

Key Words: Ethanol, Ventral tegmental area, dopamine, reward, electrophysiology, brain slice, mesencephalon

Introduction

The ventral tegmental area of Tsai (VTA) has been studied extensively in behavioral experiments in connection with the rewarding properties of drugs of abuse. Neurochemical lesions produced by agents which specifically destroy catecholamine-containing cells or nerve terminals have been used to demonstrate the importance of central monoamines in reward. Injections of the toxin 6-hydroxydopamine into the VTA[42] or into its target region the nucleus accumbens[26, 32] have been shown to reduce the rate of self-administration of psychomotor stimulants like amphetamine. Furthermore, the direct administration of opiates into the VTA appears to be rewarding[29, 30, 4]. Dopaminergic neurotransmission is necessary to sustain self-administration of drugs of abuse; dopamine antagonists like haloperidol alter the rewarding effect of opiates[10, 36, 3] and amphetamine[44]. These studies support the role of dopamine-containing neurons of the VTA in mediating the rewarding effects of at least some drugs of abuse.

Electrophysiological studies have demonstrated that a variety of drugs of abuse activate VTA neurons. *In vivo* electrophysiological studies have shown that morphine[16, 20, 25] and nicotine[22] excite VTA neurons. Like these other abused substances, ethanol has been shown to increase the firing rate of dopamine neurons of the VTA *in vivo*[12].

The *in vivo* recording methodology used in these studies has several inherent limitations. The concentration of drug which is applied to the recorded neurons cannot be precisely controlled. With systemic administration, clearance of the agent is dependent upon metabolism. In addition, it is difficult to assess whether drug effects occur directly on the recorded neuron or if drug effects on other neurons mediate this response indirectly. Local administration (pressure ejection or microiontophoresis) results in a shorter time course of drug action in a specific brain area, but the concentration of the drug is not known. For these reasons, we have utilized a brain slice preparation in order to study the effects of known concentrations of EtOH on VTA neurons under more controlled conditions.

Materials and Methods

Brain slices from Sprague-Dawley rats (100-200 gm) containing the ventral tegmental area were prepared as previously described[6]. Animals used in this study were treated in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals. Coronal sections (400 micron thick) were cut and the tissue was placed directly in the recording chamber. Small platinum weights were placed on the slice to increase the stability of recordings. The slice was covered with medium and a superfusion system then maintained the flow

of medium at 2 ml/min; the temperature in the recording chamber was kept at 35 °C. The composition of the artificial cerebrospinal fluid (aCSF) in these experiments was (in mM): NaCl 125, KCl 2.5, NaH_2PO_4 1.25, $MgSO_4$ 2, $NaHCO_3$ 25, glucose 11, $CaCl_2$ 2; the aCSF was saturated with 95% O_2/ 5% CO_2. The flow rate was continuously monitored with a flowmeter and adjustable valves were used to keep the rate constant. The small volume chamber (about 300 microliters) used in this study permitted the rapid infusion and washout of drug solutions.

In some experiments, a low calcium, high magnesium aCSF was used. In these experiments, slices were prepared in standard medium as described above. After recording from a single neuron in normal aCSF, a three-way stopcock was used to switch to aCSF identical to normal medium except that the calcium was lowered to 0.25 mM. Magnesium was then added via a calibrated pump system to medium in the fluid delivery tubing to decrease membrane excitability and restore the firing rate to the value observed in normal aCSF. This concentration of $MgSO_4$ ranged from 2 to 8 mM, so there was little difference in osmolarity between normal and low calcium aCSF. In addition, evoked responses in a hippocampal slice were used to determine whether the concentrations of calcium and magnesium used with the VTA slice were adequate to block synaptic transmission. After the low calcium/high magnesium experiment was performed with the VTA slice, a hippocampal slice was placed in the same recording chamber. A hippocampal field evoked

post-synaptic potential (fEPSP) was produced in this slice by stimulating the commissural fibers, and this response was recorded in the dendritic layer of the CA1. Once a stable response was produced, the medium was changed to the same low calcium/high magnesium aCSF that was used in the VTA experiments; abolition of the fEPSP was indication that this medium was capable of blocking synaptic transmission.

Drugs were added to the aCSF in the fluid delivery tubing by means of a calibrated infusion pump from stock solutions 100 to 1000 times the desired final concentrations. Final concentrations were calculated from aCSF flow rate, pump infusion rate and concentration of drug stock solution. Infusion of drug solutions never exceeded 2% of the flow rate of the aCSF and usually was kept below 1%. All drugs were dissolved in degassed distilled water; for concentrations above 30 mM, 95% EtOH was used in the pump. Because the pharmacologically active range for blood ethanol in the rat extends from 10-20 mM for mild motor deficits to 200 mM (lethal[17]), these studies were generally limited to application of ethanol in the range of 20 to 200 mM. In a few experiments, concentrations as high as 320 mM were tested to determine whether the response to 200 mM ethanol was maximal.

Extracellular recording electrodes were made from 1.5 mm diameter glass tubing; tip resistance of the microelectrodes ranged from 6-10 MOhm. At least one hour after preparation of the slice was

allowed for equilibration. After this period, the electrode was lowered into the VTA under visual guidance. The VTA is clearly visible in fresh tissue as a grey area medial to the substantia nigra.

Frequency of firing was determined using a window discriminator and ratemeter, the output of which was fed to a chart recorder. In addition, an IBM-PC-based data acquisition system was used to calculate, display and store the frequency of firing over 5 sec and 1 minute intervals. Differences between drug responses were determined by t-tests based upon the mean change in firing rate (normalized as a percent of control) during the peak of the drug response.

Results

Cells were selected which exhibited electrophysiological characteristics similar to those reported for presumed dopamine-containing neurons recorded *in vivo*[13,14, 41, 37, 38, 39] and *in vitro*[6, 31, 18, 19, 15]. Briefly, these neurons exhibit low firing frequency, regular firing rate, long (2.5 to 4 msec) duration extracellular action potentials, and are inhibited in their firing by moderate concentrations of dopamine. These characteristics are different from other cells found in this brain area, and these criteria for putative dopamine-containing neurons within the VTA have been well established in previous studies.

Various concentrations of EtOH in the range of 20-320 mM were tested in 45 VTA neurons from 24 preparations. EtOH increased firing of 89% of VTA neurons tested. Ethanol-induced increases in firing rate could be observed repeatedly in the same neuron. If sufficient time was allowed for recovery of the firing rate from the drug effect, a subsequent application of the same ethanol concentration produced an excitatory response of similar magnitude (Figure 1). Since VTA neurons did not show tachyphylaxis or sensitization, we were able to administer several concentrations of ethanol to the same VTA neuron, and were able to produce concentration-response curves in a single neuron (Figure 2).

Among neurons excited by EtOH, concentration-dependent increases in firing were seen for all VTA neurons tested with more than one concentration of EtOH. The mean magnitude of these increases was about 17% with 20 mM to about 53% with 160 mM EtOH and 61% with 320 mM (Figure 3). The largest excitatory response observed in a single neuron was an increase in firing rate of about 120% in response to a concentration of 160 mM EtOH. Only one neuron showed substantial inhibition of firing when tested with EtOH (50 mM). Most neurons exhibited a brief (10-30 sec) period of inhibition prior to the excitatory response.

In order to assess whether ethanol was producing its effect directly on the recorded neuron, ethanol was administered in

artificial CSF containing 0.25 mM calcium and increased magnesium, as described above. In this medium, ethanol produced a clear excitatory effect on VTA neurons (n=3; Figure 4A). To ascertain whether this low calcium/high magnesium aCSF would, in fact, block synaptic transmission, field excitatory postsynaptic potentials (fEPSPs) in the hippocampal slice were used as a bioassay for calcium-dependent synaptic transmission. In the low calcium/high magnesium aCSF used in the above experiments, the hippocampal fEPSP was completely inhibited (Figure 4B), indicating that while the ethanol response was not blocked, a synaptic response dependent on calcium could indeed be blocked.

Discussion

We have demonstrated that ethanol produces an increase in the spontaneous firing rate of VTA neurons *in vitro*. This suggests that the excitations of putative dopamine neurons in the rat VTA *in situ* reported by Gessa *et al*[12] are direct actions of EtOH on these neurons. In the behaviorally relevant range of brain concentrations of ethanol (10 - 200 mM), we observed almost exclusively excitatory effects. In addition, while there was a great deal of variability in the absolute sensitivity to ethanol among cells, each unit responded to EtOH with concentration-dependent excitation. Furthermore, this ethanol-

induced excitation reversed upon washout, and repeated administrations of the same EtOH concentration gave responses of similar magnitude.

The significance of these observations for the study of ethanol as a drug of abuse should be emphasized. Numerous other agents which support self-administration and are classified as drugs of abuse also increase the activity of mesolimbic dopamine neurons. Among these are nicotine[22, 7] and the opiates[16, 20]. Those which do not have a direct excitatory effect on VTA neurons, such as cocaine[5], increase dopaminergic tone by increasing release or blocking reuptake. In the case of cocaine, it appears as though the inhibition of reuptake in the terminal region is a more potent effect than its inhibition of firing at the level of the dopaminergic cell bodies[8, 9, 40]; this is consistent with the idea that a common feature of drugs of abuse is to increase dopaminergic outflow.

Recent investigations into the rewarding properties of EtOH have demonstrated some involvement of central dopamine systems. Pfeffer and Samson have reported that pre-treatment with dopamine antagonists such as haloperidol produces a decrease in the response rate for oral ethanol reinforcement in free feeding rats[28]. In addition, the dopamine antagonist pimozide reduces the apparent reinforcing effect of ethanol ingestion in free feeding as well as food deprived rats[27]. The fact that these observations can be made in free feeding rats

demonstrates that ethanol is rewarding independent of its nutritive qualities. These studies indicate a strong dopaminergic component in the rewarding properties of ethanol. While the involvement of other monoamine systems such as norepinephrine[1] and serotonin[23,24] have also been proposed, the behavioral studies of Pfeffer and Samson provide evidence for an independent role of dopamine in ethanol reward[27, 28].

The excitatory effect of ethanol that we have observed in the VTA is different from that seen in other brain regions studied in slice preparations. Cells of another catecholaminergic brain area, the locus coeruleus, show concentration-dependent inhibition of spontaneous firing within the same concentration range [34]. Similarly, the firing rate of cerebellar neurons *in vitro* is also reduced by ethanol[2,11]. While excitation of CNS neurons *in vitro* have been previously reported, such excitations were not concentration-dependent and/or often preceded inhibitory effects of ethanol which predominated at higher concentrations[2, 11, 34, 35].

The ability of ethanol to excite VTA neurons in medium which blocked synaptic transmission in the hippocampus suggests that this excitation is the result of an effect of ethanol directly on VTA neurons. On the other hand, GABA and dopamine release can occur in a calcium-independent manner[33, 43]. If VTA neurons are under the influence of tonic inhibition mediated by the calcium-independent release of either GABA or dopamine, then ethanol might be acting to

inhibit either GABAergic or dopaminergic neurons. It seems unlikely that the ethanol-induced excitation of putative dopamine-containing VTA neurons results from disinhibition of dopaminergic neurons as this would require that we have recorded only from those dopaminergic neurons which were not inhibited by ethanol. It is possible that ethanol inhibits GABAergic neurons in the brain slice preparation. Ethanol has been shown to inhibit neurons of the substantia nigra zona reticulata which are presumed to be GABAergic[21]. If an analogous situation occurs in the VTA, then selective inhibition of GABAergic neurons by ethanol may lead to disinhibition of VTA neurons which would account for the observed excitation. The GABAergic neurotransmission responsible for this effect would have to require very little calcium, however, since the magnitude of the ethanol response in low calcium/high magnesium media was quite similar to the magnitude in normal aCSF.

These results suggest that EtOH has a direct excitatory action on neurons that can be observed *in vitro*. These effects occur in the intoxicating range of EtOH concentrations, and are concentration-dependent, reversible, and reproducible. Experiments using intracellular recording from this *in vitro* preparation are currently in progress to determine the cellular mechanism for these responses. Because of the large body of data implicating the VTA in the rewarding properties of drugs of abuse, investigating the action of ethanol on

the neurons of the VTA may give us a broader understanding of ethanol's reinforcing effects and how ethanol abuse may be treated.

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Figure Legends

FIGURE 1

Ethanol-induced excitation of VTA neurons is reproducible. Firing rate was averaged over 10 sec intervals; the height of each vertical bar is proportional to the mean firing rate in that 10 sec interval. Horizontal bars indicate the duration of application of ethanol to this VTA neuron. Successive applications of the same concentration of ethanol produced a similar magnitude of excitation.

FIGURE 2

A. Ratemeter record of the firing rate of a single VTA neuron. Horizontal bars indicate duration of ethanol application. Firing rate was averaged over 12 second intervals; the height of each vertical bar is proportional to the mean firing rate in that 12 sec. interval. Concentrations of from 20 to 200 mM produced a concentration-dependent increase in firing rate.

B. Firing rate increase (as percent of baseline firing rate) is plotted as a function of ethanol concentration for the unit shown in part A, above.

FIGURE 3

Concentration-response curve for all VTA neurons which were excited by some concentration of ethanol. Only concentrations applied to 4 or more cells are plotted ($n_{(10mM)}$=8; $n_{(20mM)}$=10; $n_{(40mM)}$=10; $n_{(80mM)}$=28; $n_{(160mM)}$=15; $n_{(320mM)}$=4); mean values +/- S.E.M. are shown. The sigmoidal curve was fit by a least squares method; the EC_{50} calculated from these data was 98.4 +/- 4 mM.

Five cells were not excited by the highest concentrations of ethanol with which they were tested. Four of these had changes in firing rate of less than 10% ($n_{(20\ mM)}$ = 3; $n_{(60\ mM)}$ = 1), and one cell was inhibited by 50 mM ethanol (75% inhibition). In addition, one cell was inhibited by 10 mM ethanol, but was excited by 60 mM.

FIGURE 4

Ethanol-induced excitation of VTA neurons persists under conditions which block synaptic transmission.

A. Under conditions which do not support synaptic transmission, the excitatory action of ethanol is maintained. Firing rate of a single VTA neuron is shown as a rate histogram. The same concentration of ethanol was administered several times to the same neuron. During the recording, the superfusion medium was changed to one containing low calcium (0.25 mM); magnesium

chloride (7.5 mM) was infused in order to reduce the firing rate to a value close to that seen in normal medium. Under these conditions, ethanol still had an excitatory effect on this VTA neuron. Return to normal medium restored the basal firing rate, and the control response to ethanol.

B. The hippocampal fEPSP was abolished by the same low calcium/high magnesium medium as used in the experiment shown in part A above. Stimulation of commissural fibers in the hippocampal slice produces a negative-going potential which can be recorded in the dendritic layer of the CA1 region. This is thought to reflect calcium-dependent synaptic transmission. In 0.25 mM calcium/ 7.5 mM magnesium, the fEPSP was blocked. The response returned to its initial amplitude with return to medium containing normal calcium and magnesium concentrations (not shown).

Figure 1

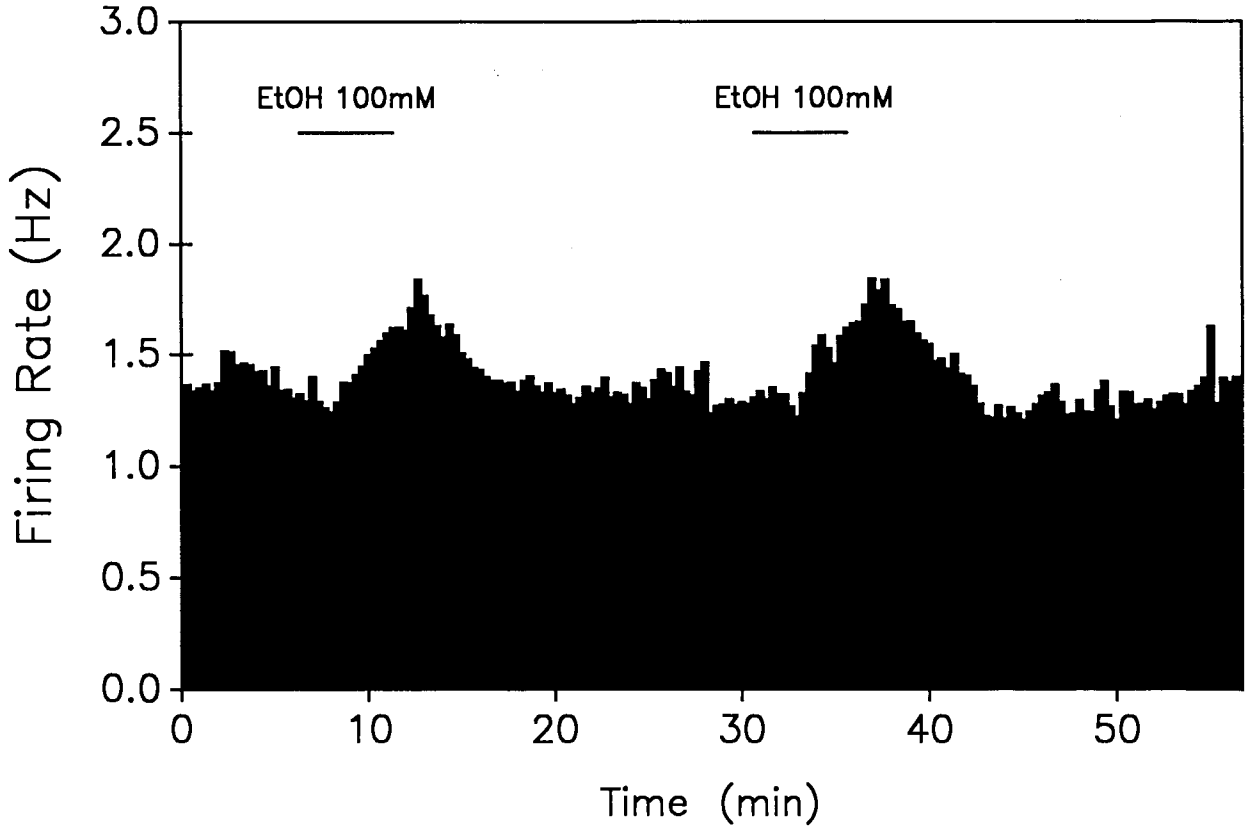

3.0
2.5
2.0
1.5
1.0
0.5
0.0
Firing Rate (Hz)
EtOH 100mM
EtOH 100mM
0
10
20
30
40
50
Time (min)

Figure 2

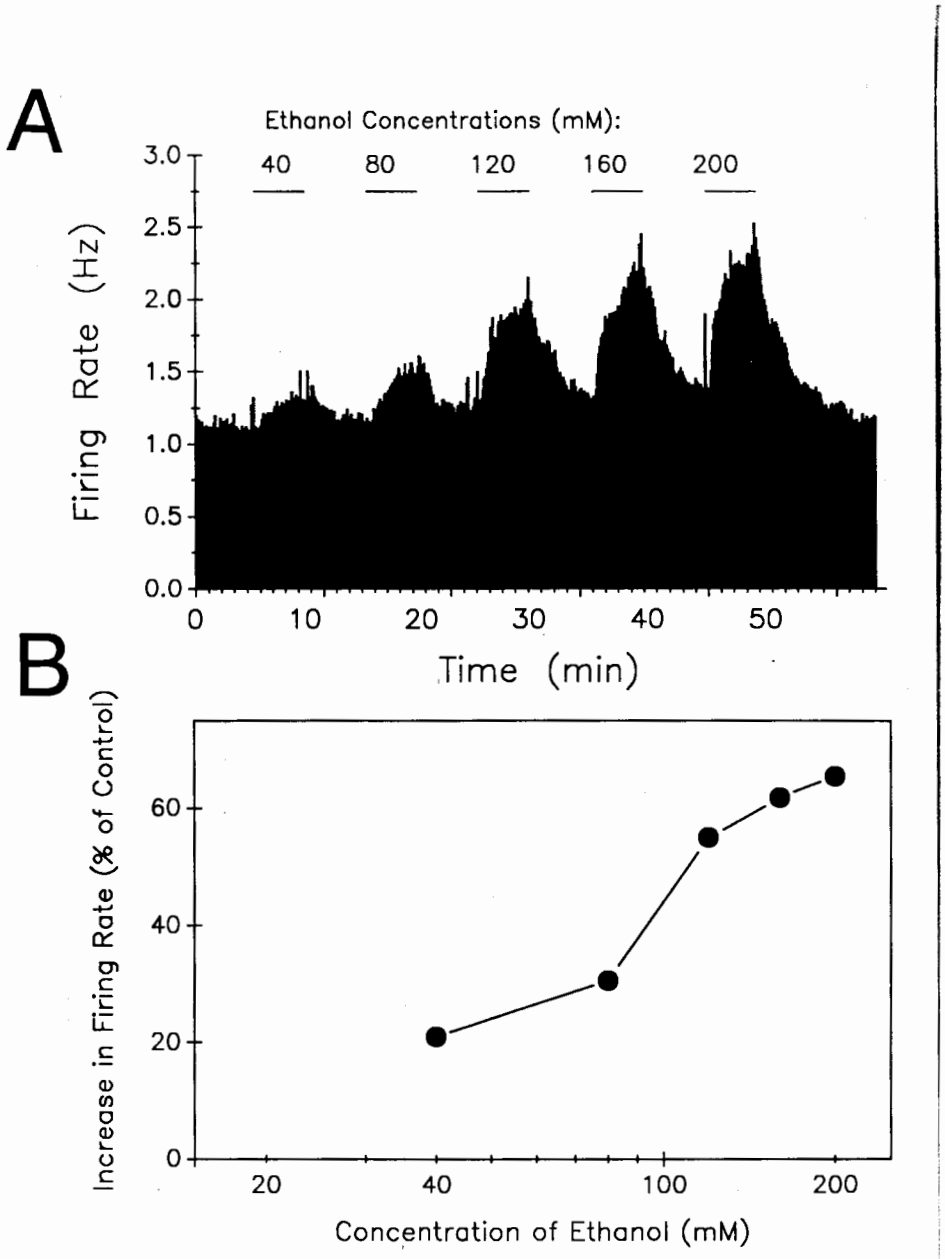

A
Ethanol Concentrations (mM):
40
80
120
160
200
Firing Rate (Hz)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
10
20
30
40
50
Time (min)
B
Increase in Firing Rate (% of Control)
60
40
20
0
20
40
100
200
Concentration of Ethanol (mM)

Figure 3

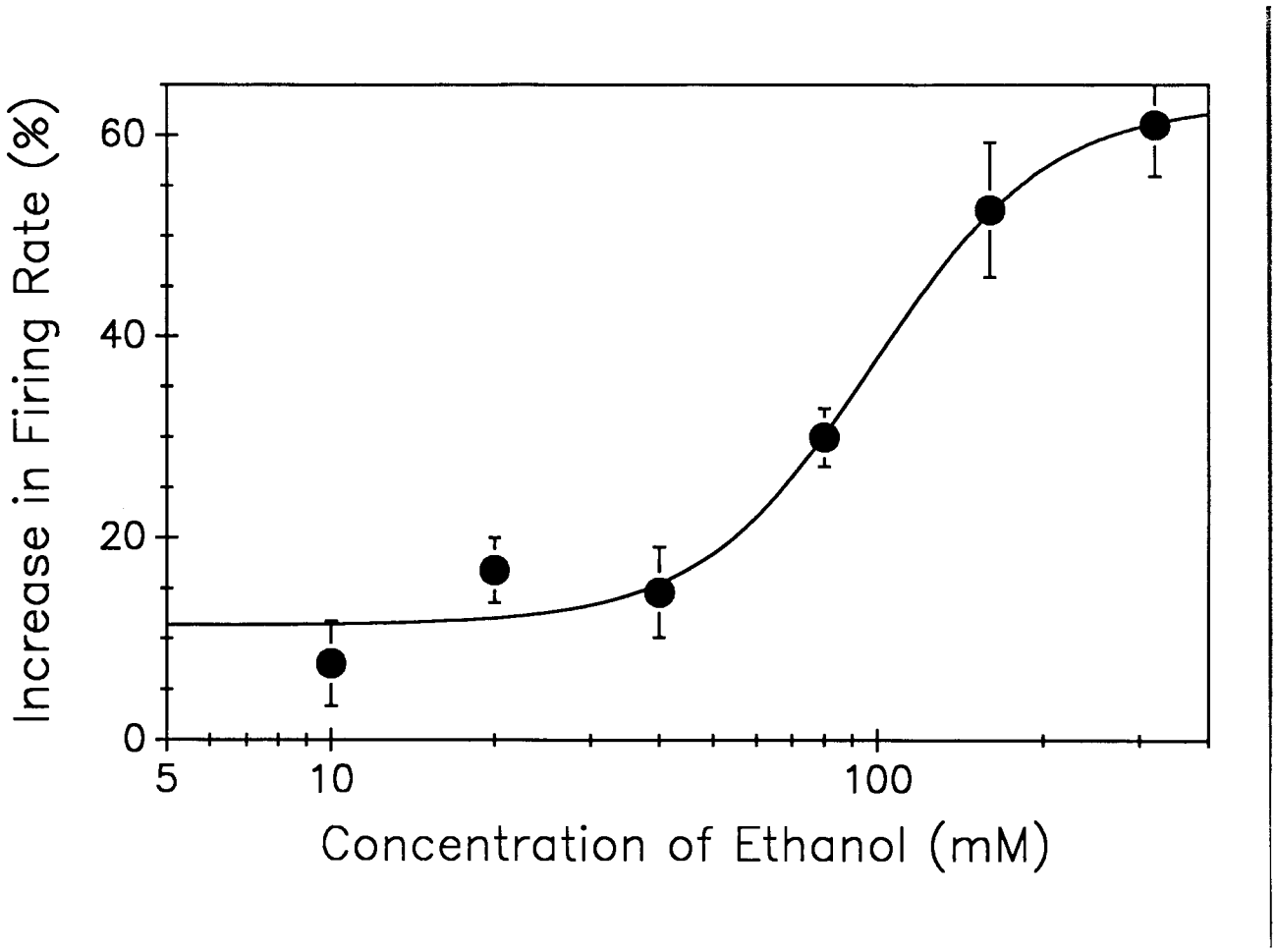
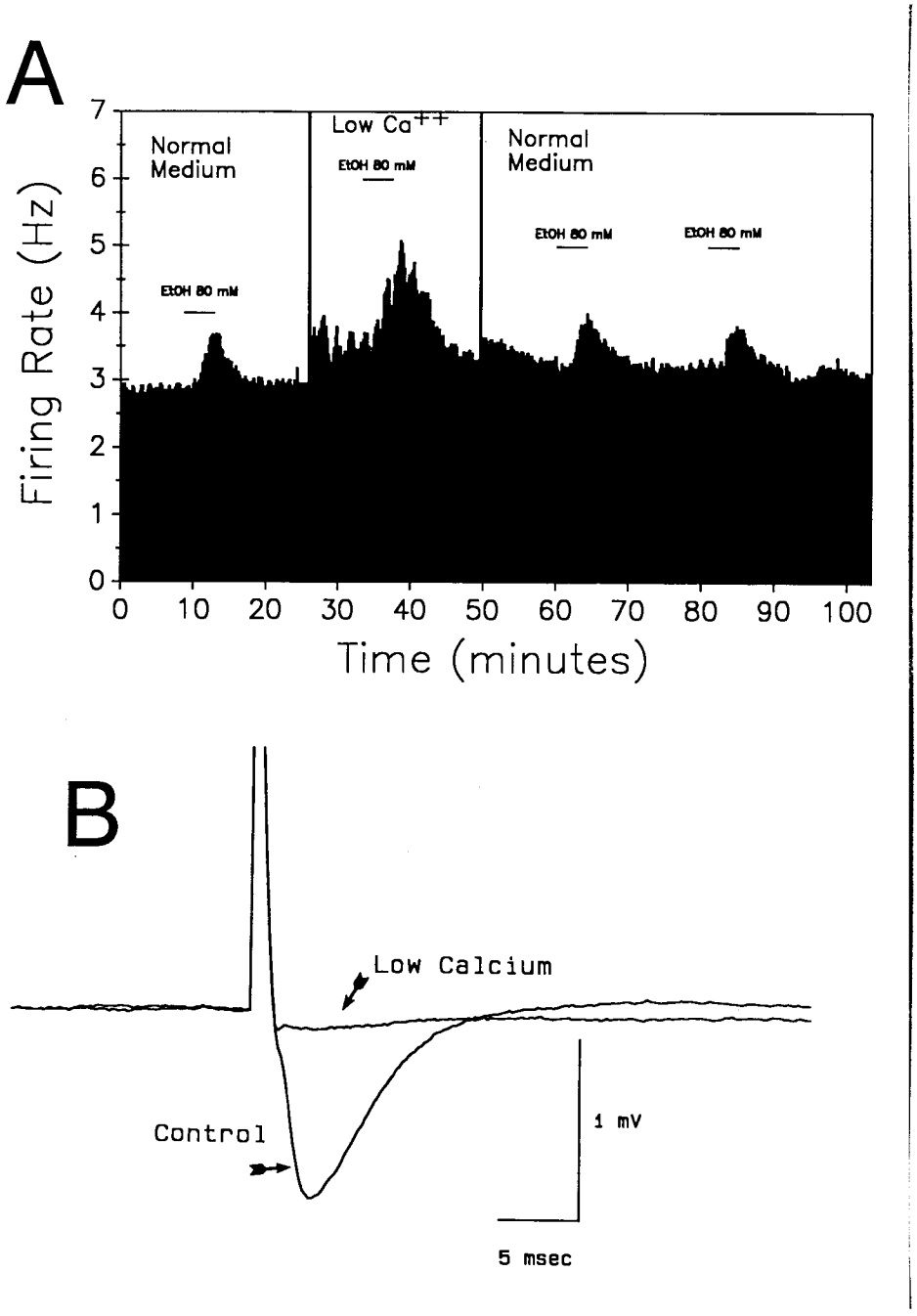

Increase in Firing Rate (%)
60
40
20
0
5
10
100
Concentration of Ethanol (mM)

Figure 4


A
Normal Medium
Low Ca++
Normal Medium
EtOH 80 mM
EtOH 80 mM
EtOH 80 mM
EtOH 80 mM
Firing Rate (Hz)
0
1
2
3
4
5
6
7
0
10
20
30
40
50
60
70
80
90
100
Time (minutes)
B
Low Calcium
Control
1 mV
5 msec