Serotonin reduces the hyperpolarization-activated current (I_h) in ventral tegmental area dopamine neurons: involvement of 5-HT_2 receptors and protein kinase C

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ABSTRACT

Dopaminergic neurons of the ventral tegmental area (VTA) have been implicated in the rewarding properties of drugs of abuse and in the etiology of schizophrenia; serotonin modulation of these neurons may play a role in these phenomena. Whole cell patch-in-the-slice recording in rat brain slices was used to investigate modulation of the hyperpolarization-activated cationic current $I_h$ by serotonin in these neurons. Serotonin (50 to 500 μM) reduced the amplitude of $I_h$ in a concentration-dependent manner; this effect was reversible after prolonged washout of serotonin. This effect was mimicked by the 5-HT$_2$ agonist $\alpha$-methylserotonin (25 μM) and reversed by the 5-HT$_2$ antagonist ketanserin (25 μM). Serotonin reduced the maximal $I_h$ current and conductance (measured at -130 mV) and caused a negative shift in the voltage-dependence of $I_h$ activation. The serotonin-induced reduction in $I_h$ amplitude was antagonized by intracellular administration of the non-specific protein kinase inhibitor H-7 (75 μM) and the selective protein kinase C inhibitor chelerythrine (25 μM). The protein kinase C activator phorbol 12, 13 diacetate (PDA, 2 μM) reduced $I_h$ amplitude; when PDA and serotonin were applied together, the effect on $I_h$ was less than additive. These data support the conclusion that serotonin reduces $I_h$ in dopaminergic VTA neurons by acting at serotonin 5-HT$_2$ receptors, which activate protein kinase C. This reduction of $I_h$ may be physiologically important, as the selective inhibitor of $I_h$, ZD7288, significantly increased dopamine inhibition of firing rate of dopaminergic VTA neurons, an effect which we previously demonstrated with serotonin.
INTRODUCTION

Dopaminergic neurons of the ventral tegmental area (VTA) have been implicated in the rewarding effects of drugs of abuse (Wise 1987) and in the etiology of schizophrenia (White and Wang 1983). Dopaminergic neurons in the VTA are the cells of origin of the mesolimbic/mesocortical dopamine pathways and provide dopaminergic innervation of the nucleus accumbens (Oades and Halliday 1987). The mesolimbic/mesocortical dopamine pathway is important for self-administration of most drugs of abuse including, cocaine, amphetamine, nicotine and ethanol (Wise 1987). The rewarding properties of some drugs of abuse, for example ethanol and nicotine, are related to their ability to excite dopaminergic cell bodies in the VTA (Di Chiara and Imperato 1988; Brodie 1991; Brodie et al. 1999b).

The ventral tegmental area receives serotonergic innervation from the raphe nuclei (Oades and Halliday 1987). Serotonin has many effects in the VTA. Serotonin acts on 5-HT_{1D} receptors to reduce GABA release from GABAergic terminals onto GABA_{h} receptors on dopaminergic neurons (Cameron and Williams 1994). Serotonin potentiates ethanol excitation (Brodie et al. 1995; Trifunovic and Brodie 1996) and dopamine inhibition (Brodie and Bunney 1996) of dopaminergic VTA neurons and both of these effects are mediated by 5-HT_{2} receptors. Cocaine also potentiates ethanol excitation of these neurons, an effect also mediated by 5-HT_{2} receptors (Bunney et al. 2000).

Dopaminergic VTA neurons have a hyperpolarization-activated cationic current (I_{h}) (Mueller and Brodie 1989; Lacey et al. 1989). I_{h} has been shown to be modulated by serotonin in a number of different types of neurons. Serotonin increases I_{h} in neurons in the thalamus (McCormick and Pape 1990), nucleus prepositus hypoglossi (Bobker and Williams 1989) and dorsal root ganglion (Cardenas et al. 1999) and these effects of serotonin are mediated by an increase in cAMP. Serotonin also increases I_{h} in motoneurons of the facial motor nucleus, but through an action at 5-
HT₂ receptors (Garratt et al. 1993). In contrast to the enhancement seen in the above studies, serotonin decreases Iₜ in cerebellar Purkinje neurons, an effect which may be mediated by 5-HT₂ receptors (Li et al. 1993).

In earlier reports on the action of serotonin on dopaminergic neurons, serotonin was observed to increase Iₜ in substantia nigra neurons (Nedergaard et al. 1991) and in 25% of VTA neurons tested (Pessia et al. 1994). In our studies, serotonin potentiation of the effects of ethanol (Brodie et al. 1995) and dopamine (Brodie and Bunney 1996), was mediated through 5-HT₂ receptors, and generally required higher serotonin concentrations or longer administrations of serotonin than were applied by others (Pessia et al. 1994). For this reason, we assessed the effects of higher serotonin concentrations on Iₜ. The present study demonstrates that serotonin reduces the amplitude of Iₜ in dopaminergic VTA neurons in a concentration-dependent manner and causes a negative shift in the voltage-dependence of Iₜ activation. Furthermore, this effect of serotonin appears to be mediated by 5-HT₂ receptors and activation of protein kinase C. Some of these results have been previously reported in abstract form (Liu et al. 1999).

METHODS

Preparation of brain slices. Brain slices containing the ventral tegmental area (VTA) were prepared from male Fischer 344 rats (90 - 150 gm) as previously described (Brodie et al. 1999a). Animals used in this study were treated in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals. Briefly, following rapid removal of the brain, the tissue was blocked coronally to contain the VTA and substantia nigra; the cerebral cortices and a portion of the dorsal mesencephalon were removed from the block. The tissue block was glued to the vibratome chuck and submerged in chilled artificial cerebrospinal fluid (aCSF). Coronal sections (400 µm thick) were
cut and the tissue was placed directly in the recording chamber (500 μl). The slice was totally submerged in aCSF maintained at a flow rate of 2 ml/min; the temperature in the recording chamber was kept at 35 °C. The composition of the aCSF in these experiments was (in mM): NaCl 126, KCl 2.5, NaH₂PO₄ 1.24, CaCl₂ 2.4, MgSO₄ 1.3, NaHCO₃ 26, glucose 11. The aCSF was saturated with 95% O₂/5% CO₂ (pH=7.4). Equilibration time of at least one hour was allowed after placement of tissue in the recording chamber before electrodes were placed in the tissue. The VTA was clearly visible in the fresh tissue as a grey area medial to the darker substantia nigra, and separated from the nigra by white matter. Recording electrodes were placed in the VTA under visual control.

**Cell identification.** Dopamine neurons have been shown to have electrophysiological characteristics very different from non-dopaminergic neurons in the mesencephalon (Lacey et al. 1989; Grace and Bunney 1984). Only those neurons which were anatomically located within the VTA and which conformed to the criteria for dopaminergic neurons established in the literature and in this laboratory (Mueller and Brodie 1989; Lacey et al. 1989) were studied. These criteria include broad action potentials, slow spontaneous firing rate (0.5 - 5 Hz) with a regular interspike interval, and the presence of time-dependent inward rectification due to the presence of the hyperpolarization-activated cationic current (Iₜᵣ).

**Whole-cell recording.** Patch electrodes were pulled from LE16 glass capillaries (Dagan, Corp, Minneapolis, MN) and the tips were fire-polished. Electrodes had resistances of 3 - 5 MΩ when filled with a solution containing (in mM): potassium gluconate 125, NaCl 15, CaCl₂ 1, MgCl₂ 2, Hepes 10, EGTA 11, ATP 3, GTP 0.3, adjusted to pH 7.3 with TRIZMA base. The electrode was advanced into the brain slice and the extracellular spontaneous action potentials of dopaminergic VTA neurons were monitored in current clamp mode. Recordings were made with conventional whole cell patch-in-the-slice methodology. Voltage clamp and current clamp recordings were
accomplished with an Axopatch-1B or Axoclamp-2A amplifier (Axon Instruments, San Rafael, CA). Current and voltage were monitored on a storage oscilloscope and on a rectilinear pen recorder and recorded on an on-line PC computer. Current and voltage steps, data acquisition and data analysis were controlled with pClamp 8 software (Axon Instruments, Foster City, CA).

Protocols and Data Analysis. In current clamp experiments, I_h was evoked by a family of hyperpolarizing current pulses (duration 800 msec); the largest current pulse was adjusted to cause a peak voltage deflection to about -120 mV. Voltage/Current (V/I) curves were constructed for peak and steady-state voltage responses. The voltage change due to I_h was measured as the difference between the peak voltage and the steady-state voltage at the end of the current pulse. In voltage clamp experiments, I_h was evoked with a family of hyperpolarizing voltage steps (duration 1 sec) from a holding potential of -60 mV to -130 mV (in 10 mV increments). Current/Voltage (I/V) curves were constructed for instantaneous and steady-state currents. The amplitude of I_h was measured as the difference between the instantaneous current measured just after the decay of the capacitive transient, and the steady-state current at the end of the voltage step; I_h / V curves were constructed from these data.

The reversal potential of I_h was determined from tail currents measured with the following voltage protocol. The membrane potential was stepped to -110 mV from a holding potential of -60 mV to activate I_h; the step duration was 1 sec which was sufficient time for full activation. The membrane potential was then stepped to test voltages between -120 mV and -10 mV (in 10 mV increments); step duration was 300 msec. The maximum amplitude of the tail current was measured just after the capacitive transient at each test voltage. Tail current amplitude was plotted as a function of the test voltage step, a curve fitted to the data points and the reversal potential was determined from the point on the curve at which the tail current reversed from outward to inward. To reduce
contamination of $I_h$ tail currents by other currents, tail current experiments were performed in the presence of tetrodotoxin (TTX, 1 $\mu$M), tetraethylammonium chloride (TEA, 2 mM), 4-amino-pyridine (2 mM) and MgCl$_2$ (20 mM) in the aCSF.

Drug-induced changes in $I_h$ were evaluated by examination of full $I/V$ and $I_h/V$ curves measured in control and in the presence of drug in the same VTA neuron. For the purposes of averaging responses from different neurons and comparisons between drug conditions, drug-induced changes in $I_h$ were summarized as the percent decrease in $I_h$ amplitude measured at -120 mV. Percent decrease in $I_h$ was calculated as $((A_D - A_C) / A_C) \times 100$, where $A_D$ is the amplitude of $I_h$ measured in the presence of the drug and $A_C$ is the amplitude of $I_h$ measured in control medium in the same cell prior to drug administration. Averaged data are expressed as mean ± S.E.M. Statistical significance of data from different drug conditions was assessed with a Student’s t-test, one way ANOVA or two way ANOVA as appropriate. When needed, the Student-Newman-Keuls post hoc test was used to test all pairwise multiple comparisons and the Dunnett's post hoc test was used for multiple comparisons versus the control group.

**Extracellular recording.** Extracellular recording electrodes were made from 1.5 mm diameter glass tubing with filament and were filled with 0.9% NaCl. Tip resistance of the microelectrodes ranged from 4 - 8 MΩ. The Fintronics amplifier used in these recordings includes a window discriminator, the output of which was fed to both a rectilinear pen recorder, and a computer-based data acquisition system which was used for on-line and off-line analysis of the data. The multiplexed output of the Fintronics amplifier was displayed on an analog storage oscilloscope, for accurate adjustment of the window levels used to monitor single units. An IBM-PC-based data acquisition system was used to calculate, display and store the frequency of firing over 5 second and 1 minute intervals. Firing rate was determined before and during drug application. Firing rate was
calculated over a 1 minute interval immediately prior to drug administration and a 1 minute interval during the peak drug effect; drug-induced changes in firing rate were expressed as the percentage change from the control firing rate according to the formula 
\[
\frac{(FR_D - FR_C)}{FR_C} \times 100
\]
where \(FR_D\) is the firing rate during the peak drug effect and \(FR_C\) is the control firing rate. The change in firing rate thus is expressed as a percentage of the initial firing rate, which controls for small changes in firing rate which may occur over time.

**Drugs.** The flow rate of fluid to the recording chamber was continuously monitored with a flowmeter, and adjustable valves were used to keep the rate constant. The small volume chamber used in this study permitted the rapid application and washout of drug solutions. Because the slice was submerged in ACSF in the recording chamber, applied agents reach equilibrium in the chamber quickly (2 to 3 min). 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. The addition of drug solutions to the aCSF was performed in such a way as to permit the drug solution to mix completely with aCSF before this mixture reached the recording chamber. Final concentration was calculated from aCSF flow rate, pump infusion rate, and concentration of drug stock solution. Effects on \(I_h\) were measured at 5 and 10 min after bath application of serotonin. In general, the effect on \(I_h\) was greater at 10 min and therefore the data shown in this paper were collected at this time point. Dopamine was given for six minutes or less if firing rate was completely blocked. ZD7288 was given for 20 minutes before dopamine was retested. Most other drugs were administered by bath application as described above and their effects on \(I_h\) were quantitated after 10 min of application. The exceptions to this were H-7, chelerythrine, and cAMP, which were added to the internal solution and were applied intracellularly from the recording pipette. In this case, a period of 10-20 min was allowed after the rupture of the patch to permit the drug to enter the cell.
Serotonin creatinine sulfate complex, α-methylserotonin maleate, ketanserin tartrate, dopamine hydrochloride, and all other drugs used in this study were obtained from Sigma/RBI (St. Louis, MO) except for H-7 and dibutryl-cAMP which were obtained from Tocris Cookson, (Ballwin, MO); tetrodotoxin (TTX), which was used only in the tail-current experiments, was obtained from Calbiochem (La Jolla, CA).

RESULTS

Serotonin reduces $I_h$: current-clamp studies

Initially, $I_h$ was studied in current clamp mode in a total of 21 VTA neurons with whole cell patch-in-the-slice recording. All cells met the criteria for dopaminergic VTA neurons described in the Methods section. At the resting membrane potential these cells fired overshooting spontaneous action potentials. When tested with a series of hyperpolarizing current pulses 800 msec in duration (Fig. 1A, upper panel), all of these neurons exhibited a robust time-dependent depolarizing “sag” in voltage responses more negative than about -70 mV (Fig. 1A, lower panel), due to activation of $I_h$. The difference between the peak and steady-state voltage response indicates the magnitude of $I_h$. In the presence of 300 μM serotonin, the difference between the peak and steady-state voltage response was reduced, showing that $I_h$ is reduced by serotonin in these dopaminergic VTA neurons (Fig. 1B). Figure 1C is a Voltage/Current (V/I) curve in which the voltage responses shown in Figs. 1A and 1B are plotted as a function of the negative current pulses used to evoke them. This graph shows that serotonin produced a relatively small change in the peak voltage response, but produced a large reduction in the steady-state response. Figure 1D plots the difference between the peak and steady-state voltage responses (magnitude of $I_h$), as a function of the peak voltage attained. This graph clearly shows the reduction in $I_h$ over the voltage range from about -70 mV to -120 mV.
The serotonin-induced reduction of $I_h$ in dopaminergic VTA neurons was concentration-dependent ($n=21$). Figure 2 illustrates the effect of 4 different concentrations of serotonin tested on a dopaminergic VTA neuron. Figure 2A is a V/I curve showing the effect of serotonin on the peak and steady-state components of the voltage responses in this neuron and Fig. 2B shows the voltage difference between peak and steady-state as a measure of the magnitude of $I_h$ in the same neuron. Serotonin (50 - 500 μM) caused a concentration-dependent reduction in the steady-state current, but little change in the peak voltage (Fig. 2A). Figure 2B shows that this corresponds to a concentration-dependent reduction in the magnitude of $I_h$. In all nine cells tested with more than one serotonin concentration (100 - 500 μM) in current clamp experiments, higher concentrations of serotonin produced a larger decrease in $I_h$ than the lower concentrations. Table 1 shows the effects of serotonin (50 to 500 μM) on 21 dopaminergic VTA neurons; with concentrations of 100 μM or higher, more than 90% of the neurons showed a reduction in $I_h$. No cell exhibited a serotonin-induced increase in $I_h$, but a few showed no change in $I_h$, including 4 cells tested with 25 μM serotonin.

For the population of cells tested with 300 μM serotonin ($n=15$), serotonin did not significantly change the membrane potential (paired t-test, $p>0.05$) or the input resistance (paired t-test, $p>0.05$). Specifically, prior to serotonin administration, the mean membrane potential was $-51.3 \pm 1.1$ mV and the mean input resistance was $291 \pm 52$ MΩ; in the presence of 300 μM serotonin, the mean membrane potential was $-50.2 \pm 1.2$ mV and the mean input resistance was $319 \pm 46$ MΩ.
As has been previously shown (Cathala and Paupardin-Tritsch 1997; Jiang et al. 1993), the I_h measured in this study was blocked by addition of cesium to the external medium. Cesium (2.5 mM) caused complete block of I_h in 5 of 9 dopaminergic VTA neurons, and partial block in the remaining 4 neurons (data not shown). I_h was completely blocked by 5 mM cesium (Appel et al. 2003).

Voltage clamp characterization of the serotonin-induced reduction of I_h

Following the current-clamp studies described above, the effect of serotonin on I_h was further characterized with whole cell voltage-clamp recording in a total of 40 dopaminergic VTA neurons in brain slices. Voltage clamp recording allowed the effect of serotonin on I_h to be studied at constant voltage and allowed determination of the effect of serotonin on the voltage-dependence of activation of I_h (see Fig. 7, below). The mean membrane potential of these neurons (measured in current clamp mode) was -44.4 ± 0.5 mV, and the mean input resistance was 108.6 ± 4.5 MΩ (n=40). Figure 3A illustrates the currents recorded in response to a series of negative voltage steps (duration 1 sec) from a holding potential -60 mV to -130 mV (in 10 mV increments). Following the capacitive artifact, there was a time-dependent increase in inward current reflecting the activation of I_h (lower panel). The instantaneous current (ins.) (arrow) was measured just after the decay of the capacitive transient and the steady-state current (ss.) (arrow) was measured near the end of the voltage command. Figure 3B shows that in the presence of 500 µM serotonin, the steady-state inward current was substantially reduced in comparison to control (Fig 3A), reflecting a serotonin-induced decrease in I_h. Figure 3C is a Current/Voltage (I/V) curve of the instantaneous and steady-state currents plotted as a function of the step voltage, in control (data from Fig. 3A) and in the presence of 500 µM serotonin (data from Fig. 3B). Note that serotonin decreased the steady-state current at all voltage steps more
negative than \(-70\) mV but had little or no effect on the instantaneous current. In Fig. 3D, the difference between the instantaneous and steady-state current (amplitude of \(I_h\)) is plotted versus the step voltage, in the absence and presence of 500 \(\mu\)M serotonin. Note that \(I_h\) exhibited inward rectification and was activated at potentials more negative than \(-70\) mV. The large reduction in \(I_h\) caused by the addition of 500 \(\mu\)M serotonin can be clearly seen.

[Figure 3 about here]

Figure 4 shows the concentration-dependence of serotonin reduction of \(I_h\) under voltage clamp conditions. Fig. 4A is an I/V curve showing the instantaneous and steady-state currents plotted as a function of the hyperpolarizing voltage step; the currents were measured in control and after bath applications of three different concentrations of serotonin (100, 300 and 500 \(\mu\)M) to the same neuron. Serotonin (100 \(\mu\)M to 500 \(\mu\)M) decreased the steady-state current in a concentration-dependent manner, but did not affect the instantaneous current. The difference between the instantaneous and steady-state current, \(I_h\), is plotted as a function of voltage in Fig. 4B; the concentration-dependence of the reduction of \(I_h\) by serotonin can be seen clearly in this graph. In all five cells tested with more than one serotonin concentration (100 \(\mu\)M or greater) in voltage clamp experiments, higher concentrations of serotonin produced a larger decrease in \(I_h\) than the lower concentrations. A concentration-response curve for the mean serotonin-induced reduction of \(I_h\) in voltage-clamp experiments is illustrated in Fig. 4C. The effect of serotonin was concentration-dependent (one way ANOVA, \(F=12.47, df=2, 36, p<0.001\)).

[Figure 4 about here]

[Table 2 about here]

Table 2 shows that serotonin (30 to 500 \(\mu\)M) reduced \(I_h\) in most of the 40 cells tested under voltage clamp conditions. With serotonin concentrations of 50 \(\mu\)M or higher, more than 90\% of the
neurons showed a reduction in $I_h$, no neurons exhibited an increase in $I_h$, and two neurons showed no change. By contrast, 30 µM serotonin (tested on 16 neurons) produced an increase in $I_h$ in 37.5% of the cells, a decrease in about 37.5%, and no change in the remaining 25% of the neurons.

When the recording could be held for sufficient time, the effect of serotonin was completely reversible. Fig. 5 illustrates such an experiment. In this neuron, 300 µM serotonin clearly reduced the amplitude of $I_h$, and following a washout period of one hour, $I_h$ returned to the pre-serotonin levels. Partial or total recovery upon washout was observed in six neurons. For the six neurons tested, before serotonin administration $I_h$ at -130 mM was $288 \pm 0.05$ pA, in the presence of serotonin, $I_h$ at -130 mV was $-202 \pm 0.05$ pA, and upon washout of from 20 to 60 min, $I_h$ at -130 mV was $-279 \pm 0.05$ pA. Serotonin significantly reduced $I_h$ amplitude (one-way repeated measures ANOVA, $F = 5.60$, df = 2, 10; $p < 0.05$), but there was no significant difference in $I_h$ at -130 mV between control and washout values (Student-Newman-Keuls, $p > 0.05$).

[Figure 5 about here]

**The effect of serotonin is mimicked by α-methylserotonin**

The effect of serotonin to reduce $I_h$ was seen primarily at concentrations higher than 50 µM. One reason for this could be efficient serotonin reuptake in the brain slice preparation, which would reduce the effective concentration of serotonin at the receptor. We have previously demonstrated serotonergic effects on ethanol potency with low concentrations of the reuptake inhibitors cocaine and cocaethylene in the absence of exogenous serotonin (Bunney et al. 2000; Bunney et al. 2001), indicating that both release of serotonin and active reuptake of endogenous serotonin occur in our slice preparation. Because the results of experiments using reuptake blockers in combination with exogenous serotonin would be difficult to interpret due to unknown levels of endogenous serotonin, we used a serotonin analog which is not a substrate for reuptake, α-methylserotonin ($\alpha$-Me5-HT).
Figure 6 illustrates the results of voltage-clamp experiments to assess the effects of \(\alpha\)-Me5-HT on \(I_h\). In the I/V curve shown in Figure 6A, \(\alpha\)-Me5-HT (25 \(\mu\)M) caused a clear decrease in \(I_h\) compared to control; \(\alpha\)-Me5-HT (25 \(\mu\)M) decreased \(I_h\) in all 8 dopaminergic VTA neurons tested. Specifically, \(\alpha\)-Me5-HT (25 \(\mu\)M) significantly reduced the mean \(I_h\) amplitude measured at -120 mV from -281 ± 26 pA in control, to -229 ± 29 pA in \(\alpha\)-Me5-HT (paired t-test, \(t = -7.69, df = 7, p < 0.001\)). The mean percent decrease in \(I_h\) amplitude caused by 25 \(\mu\)M \(\alpha\)-Me5-HT was -19.7 ± 2.8\% (n=8), which was similar in magnitude to the mean percent decrease caused by 100 \(\mu\)M serotonin -22.1 ± 4.3\% (n=5).

This effect of \(\alpha\)-methylserotonin was reversible after 30 to 40 min of washout (Fig. 6B) in all three dopaminergic VTA neurons tested. Specifically, \(\alpha\)-Me5-HT (25 \(\mu\)M) significantly decreased the mean \(I_h\) amplitude measured at -120 mV from -258 ± 5 pA in control to -197 ± 6 pA in \(\alpha\)-Me5-HT, and then fully reversed with washout to -251 ± 16 pA (one way repeated measures ANOVA, \(F = 14.64, df = 2, 4, p = 0.014\), Student-Newman-Keuls post hoc tests, \(p<0.05\)).

The effect of \(\alpha\)-methylserotonin is reversed by ketanserin

Since \(\alpha\)-methylserotonin is a selective agonist for the 5-HT\(_2\) receptor, the above results suggest that the 5-HT\(_2\) serotonin receptor mediates the inhibitory effect of serotonin on \(I_h\). To further establish this, we tested whether ketanserin, a selective 5-HT\(_2\) antagonist, could reverse the reduction of \(I_h\) by \(\alpha\)-methylserotonin; this was found to be the case in all three dopaminergic VTA neurons tested. Specifically, \(\alpha\)-Me5-HT (25 \(\mu\)M) significantly decreased the mean \(I_h\) amplitude measured at -120 mV from -354 ± 39 pA in control, to -302 ± 55 pA in \(\alpha\)-Me5-HT. When ketanserin (25 \(\mu\)M) was added to the superfusate in the continued presence of \(\alpha\)-Me5-HT, the effect of \(\alpha\)-Me5-HT was fully reversed. In the presence of both ketanserin and \(\alpha\)-Me5-HT, the mean \(I_h\) amplitude was -393...
± 37 pA, which was significantly larger than in the presence of α-Me5-HT alone (one-way repeated measures ANOVA, F= 21.44, df = 2, 4, p = 0.007, Student-Newman-Keuls post hoc tests p < 0.05).

Serotonin causes a negative shift in the voltage-dependence of $I_h$ activation

$I_h$ was measured in 10 dopaminergic VTA neurons in the absence and presence of 300 μM serotonin. The voltage clamp protocol and data analysis was the same as described for Fig. 3 above. $I_h$ currents were converted to conductance ($G_h$) with the equation $G_h = \frac{I_h}{(E - E_{rev})}$, where E is the voltage step used to evoke the current and $E_{rev}$ is the reversal potential of $I_h$. The values for $E_{rev}$ were -39 mV for control and -42 mV for serotonin, as determined by tail current analysis in three cells (see Methods). The mean maximal $G_h$ conductance ($G_h$ measured at -130 mV) was 3.3 ± 0.4 nS in control and 1.7 ± 0.2 nS in 300 μM serotonin (n=10), or a mean percent reduction in maximal conductance of -47% (with each cell serving as its own control). This serotonin-induced reduction in maximal $G_h$ was significant (paired t-test, t= 4.75, df=9, p=0.001).

The conductance ($G_h$) for each cell was normalized ($G_h/G_{max}$), where $G_{max}$ was taken to be the value of $G_h$ at -130 mV; this was done to permit the pooling of responses from the 10 neurons, as shown in Fig. 7. The mean normalized conductance ($G_h/G_{max}$) in the absence and presence of serotonin (300 μM) is plotted as a function of voltage. Serotonin shifted this conductance/voltage curve in the negative direction, indicating a negative shift in the voltage-dependence for activation of $G_h$. The serotonin-induced shift was statistically significant (two-way ANOVA, F=10.69, df: 1, 126, p=0.001). The effect of voltage was also significant (F=167.57, df = 6, 126, p<0.001). The smooth curves on Fig. 7 were fit to the data points with the Boltzmann equation of the form

$$\frac{G_h}{G_{max}} = \frac{1}{1 + e^{(V-V_{1/2})/k}}$$
The half-activation voltage \((V_{1/2})\) determined from the Boltzmann equation was \(-88 \pm 0.6\) mV in control and \(-95 \pm 1.6\) mV in 300 µM serotonin, a negative shift of about 7 mV (Fig. 7). The slope factor \((k)\) was similar in control \((12 \pm 0.6)\) and in serotonin \((11 \pm 1.5)\).

**Effect of cAMP agonists and protein kinase inhibitors on the serotonin-induced reduction of \(I_h\)**

Drug-induced changes in \(I_h\) were evaluated by examination of full \(I/V\) and \(I_h/V\) curves measured in control and in the presence of drug in the same VTA neuron. For the purposes of averaging responses from different neurons and comparisons between drug conditions, drug-induced changes in \(I_h\) were summarized as the percent decrease in \(I_h\) amplitude measured at -120 mV (see Methods for formula). We first examined whether increasing intracellular cAMP would alter the serotonin-induced reduction of \(I_h\). Bath application of serotonin alone (300 µM) caused a mean percent decrease in \(I_h\) amplitude of \(-39.1 \pm 5.6\%\) in 12 dopaminergic VTA (Fig. 8, far left bar). In another group of dopaminergic VTA neurons, 1 mM cAMP was included in the recording pipette. A small increase in the amplitude of \(I_h\) was seen after 10-15 min of recording in all 5 of these neurons, indicating that cAMP had diffused into the cell and was exerting a positive modulatory effect on \(I_h\). Figure 8 shows that after 10-15 min of intracellular administration of 1 mM cAMP, bath application of 300 µM serotonin still reduced \(I_h\) amplitude causing a mean percent decrease of \(-32.5 \pm 7.1\%\) \((n=5)\). The effect of serotonin was also tested after 10 min of bath application of the membrane permeable cAMP analogue dibutyryl-cAMP. Serotonin (300 µM) also reduced \(I_h\) amplitude when applied in the presence of 500 µM dibutyryl-cAMP \((-27.5 \pm 4.7\%, \ n = 3)\).

The involvement of protein kinases A and C in the serotonin-induced reduction in \(I_h\) was tested with the non-specific protein kinase inhibitor H-7 (Hidaka et al. 1984) and the selective...
protein kinase C inhibitor chelerythrine (Herbert et al. 1990). When the non-specific protein kinase inhibitor H-7 (75 μM) was included in the recording pipette, there was no change in $I_h$ amplitude after 20 min of recording (n=4). Subsequent bath application of 300 μM serotonin caused only a very small mean percent decrease in $I_h$ amplitude at -120 mV (-4.4 ± 11.0%, n=4). Likewise, when the protein kinase C inhibitor chelerythrine (25 μM) was included in the recording pipette, there was no consistent change in $I_h$ amplitude after 10 min of recording (n=7). Subsequent application of 300 μM serotonin reduced $I_h$ amplitude measured at -120 mV by -16.2 ± 3.8%, n=7), a much smaller reduction than the effect of serotonin alone. There was a significant difference between the drug conditions shown in Fig. 8 (one way ANOVA, $F = 4.2$, df: 4, 26, $p = 0.009$) and Dunnett’s post hoc comparisons demonstrated that the effect of serotonin in the presence of internal cAMP or external dibutyryl-cAMP was not significantly different from serotonin alone ($p>0.05$), but there was a significant difference between the control effect of serotonin alone and the effect of serotonin in the presence of H-7 or chelerythrine ($p < 0.05$). Since both H-7 and chelerythine significantly attenuated the serotonin-induced reduction of $I_h$ and both are potent inhibitors of protein kinase C, the following experiments with the phorbol ester, phorbol 12, 13-diacetate (PDA), were undertaken to further examine the possible involvement of protein kinase C.

[Figure 9 about here]

**The protein kinase C activator phorbol 12, 13-diacetate (PDA) mimics and occludes the serotonin-induced reduction in $I_h$**

Figure 9A shows a current/voltage (I/V) curve of instantaneous and steady-state currents as a function of the step voltage in control and in the presence of 2 μM PDA. DMSO (0.1%) was used as a vehicle for the PDA, and was present both in control and in the PDA-containing media. Like
the effect of serotonin shown in Fig. 3C, PDA decreased the steady-state current but had little or no
effect on the instantaneous current. In Figure 9B, the difference between the instantaneous and the
steady-state currents (I_h amplitude) is plotted versus the step voltage in the absence and presence of
2 μM PDA. Note the large reduction in I_h amplitude in the presence of PDA which closely resembles
the effect of serotonin shown in Fig. 3D. Similar effects on I_h current-voltage (I/V) curves were
observed in all 3 dopaminergic VTA neurons tested with 2 μM PDA.

Figure 10 compares the effect on I_h in dopaminergic VTA neurons of DMSO vehicle alone,
serotonin (5-HT) in DMSO, PDA in DMSO, and 5-HT and PDA applied together in DMSO. Bars
indicate the mean percent decrease in I_h measured at -120 mV (see Methods for formula). The far
left bar shows that addition of 0.1% DMSO to the aCSF did not change I_h amplitude (-1.7 ± 5.0%,
n=7). DMSO (0.1%) was present in the aCSF for the other three experimental groups shown
(remaining 3 bars). When tested in the presence of DMSO, 300 μM serotonin caused a mean percent
decrease in I_h of -35.3 ± 9.1% (n=4). Bath application of PDA (2 μM) in DMSO vehicle also reduced
I_h amplitude (-44.9 ± 7.8 %, n=3). When PDA (2 μM) and serotonin (300 μM) were applied together
(in DMSO), the mean percent decrease in I_h amplitude was -51.5 ± 20.0 % (n=3). Note that this
effect was much less than the sum of the effects of serotonin and PDA when tested alone. Asterisks
indicate a significant difference from the effect of DMSO vehicle alone. (One way ANOVA, F=6.72,
df= 3, 13, p=0.006; Student-Newman Keuls post hoc tests p < 0.02). The mean percent decreases
in I_h measured in 5-HT, PDA and 5-HT plus PDA were not significantly different from each other
(p>0.05).
Reduction of $I_h$ with ZD7288 potentiates dopamine inhibition

Since $I_h$ is activated at membrane potentials negative to -70 mV, and the resting membrane potential of dopaminergic neurons is between -55 and -40 mV (Grace and Bunney 1983; Lacey et al. 1987), the physiological relevance of reduction of $I_h$ by serotonin was examined. We previously have demonstrated that serotonin potentiates dopamine inhibition of the spontaneous firing rate of dopaminergic VTA neurons through an action at 5-HT$_2$ receptors (Brodie and Bunney 1996). If serotonin potentiates dopamine inhibition through a reduction of $I_h$, then selective blockade of $I_h$ also would be expected to potentiate dopamine inhibition. ZD7288 is a selective blocker of $I_h$ (Seutin et al. 2001). The spontaneous firing rate of dopaminergic VTA neurons was measured with extracellular single unit recording (see Methods). Figure 11A and B illustrates the effect of 30 µM ZD7288 on dopamine inhibition of a single dopaminergic VTA neuron. For the population of cells tested before and in the presence of 30 µM ZD7288 (n=14), dopamine produced concentration-dependent decrease in firing rate (two-way ANOVA, F=12.42, df=4,76; p < 0.001). ZD7288 alone increased the basal firing rate by 11.0 ± 5.2%. The responses to dopamine (1 to 10 µM) were much larger in the presence of 30 µM ZD7288 than prior to ZD7288 addition in these cells (Fig. 11C); dopamine inhibition was significantly increased in the presence of ZD7288 compared to the control condition (two-way ANOVA, F=31.591, df=1,76; p < 0.001).

DISCUSSION

The present current clamp and voltage clamp study demonstrates that serotonin (50-500 µM) reduces the amplitude of the hyperpolarization-activated cationic current ($I_h$) in dopaminergic VTA neurons. This reduction was concentration-dependent and reversible with washout of serotonin. $I_h$ was also reduced by the 5-HT$_2$ agonist α-methylserotonin and this effect was reversed by the 5-HT$_2$
antagonist ketanserin. The maximal $I_h$ current (measured at -130 mV) was reduced by serotonin, as was the maximal conductance ($G_h$). The normalized conductance ($G_h/G_{max}$) curve showed that serotonin caused a negative shift in the voltage-dependence of activation of $I_h$. The serotonin-induced reduction in $I_h$ amplitude was blocked by the non-specific protein kinase inhibitor H-7 and the selective protein kinase C inhibitor chelerythrine, which suggests that it was mediated by protein kinase C. Consistent with this interpretation, the protein kinase C activator PDA mimicked and occluded the serotonin-induced reduction in $I_h$ amplitude. Specifically, PDA applied alone reduced $I_h$ amplitude. When serotonin and PDA were applied together, the effect on $I_h$ was less than additive, suggesting that they were reducing $I_h$ by a common mechanism. Taken together, these data demonstrate that serotonin reduces $I_h$ in dopaminergic VTA neurons and support the conclusion that this effect is mediated by 5-HT$_2$ receptors and protein kinase C.

The present study demonstrates that serotonin concentrations of 50 to 500 µM consistently decrease $I_h$ in dopaminergic neurons of the VTA, whereas serotonin concentrations below 50 µM, produced mixed effects. For example, our current clamp experiments with 25 µM serotonin showed no change in $I_h$. Our voltage clamp experiments testing 30 µM serotonin in 16 VTA neurons showed that 37.5% of cells showed a decrease in $I_h$, but an equal proportion showed an increase in $I_h$ with this lower serotonin concentration; the remaining 25% of neurons showed no change. The observation that a lower concentration of serotonin can increase $I_h$ in some dopaminergic VTA neurons is consistent with earlier reports that 40 µM serotonin increased $I_h$ in dopaminergic neurons in the substantia nigra (Nedergaard et al. 1991) and that serotonin (30 - 100 µM) increased $I_h$ in 25% of dopaminergic VTA neurons tested (Pessia et al. 1994). It should be noted, however, that in the present study increases in $I_h$ were never observed with serotonin concentrations of 50 µM or higher, whereas the decrease in $I_h$ was clearly concentration-dependent.
In view of estimates that the concentration of serotonin released at the synapse may reach the mM range (Bunin and Wightman 1998), it is likely that the effects of the relatively high concentrations of serotonin (50 to 500 μM) observed in the present study are physiologically relevant. Furthermore, high concentrations of serotonin, such as were used in these studies, should have a faster onset of action than lower concentrations. In addition, serotonin reuptake blockers were not added to the superfusion medium, and given active reuptake of serotonin present in brain slices, it is likely that the effective concentration of 5-HT at the receptor was lower than the concentration applied in the bath in our experiments. This is supported by the fact that 25 μM α-methylserotonin, a 5-HT<sub>2</sub> agonist which is not a substrate for reuptake, was sufficient to significantly reduce I<sub>h</sub> amplitude. The size of the reduction in I<sub>h</sub> by 25 μM α-methylserotonin was very similar to the reduction by 100 μM serotonin, indicating the same effect could be produced by a 4-fold lower concentration of α-methylserotonin.

The effectiveness of the 5-HT<sub>2</sub> agonist α-methylserotonin to reduce I<sub>h</sub>, and the reversal of this effect by the 5-HT<sub>2</sub> antagonist ketanserin, suggest that serotonergic reduction of I<sub>h</sub> is mediated by serotonin 5-HT<sub>2</sub> receptors. An ultrastructural study has shown that 5-HT<sub>2A</sub> receptors are present on the dendrites and somata of dopaminergic VTA neurons (Doherty and Pickel 2000). Since the action of serotonin at 5-HT<sub>2</sub> receptors is transduced by the phosphatidylinositol signal transduction system (Conn and Sanders-Bush 1984), the identity of the receptor as 5-HT<sub>2</sub> is consistent with the antagonism of the effect of serotonin by protein kinase C inhibitor chelerythrine and the less selective protein kinase inhibitor H-7, and by the mimicking of the inhibitory effect of serotonin on I<sub>h</sub> by PDA. Taken together, these results support the hypothesis that serotonin, by acting at a 5-HT<sub>2</sub> receptor, causes the activation of protein kinase C, which then reduces I<sub>h</sub> amplitude. Additional
studies will be necessary to determine how activation of protein kinase C reduces $I_h$ amplitude in dopaminergic VTA neurons.

The serotonin-induced inhibition of $I_h$, observed in the present study in dopaminergic VTA neurons, has also been reported in cerebellar Purkinje cells (Li et al. 1993). The effect of serotonin in Purkinje cells closely resembles the effect in dopaminergic VTA neurons described in the present paper. In both studies, serotonin induced a similar negative shift in the voltage-dependence of activation of $I_h$. Interestingly, the serotonin-induced inhibition of $I_h$ in Purkinje neurons was mimicked by the 5-HT$_2$ agonist 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) (Li et al. 1993).

In contrast, in most studies in the literature, serotonin-induced increase of $I_h$ has been observed. For example, serotonin increases $I_h$ in inferior olivary neurons, and this increase is mediated by 5-HT$_2$ receptors, as it is blocked by 5-HT$_2$ antagonist ketanserin and is mimicked by 5-HT$_2$ agonist DOI (Placantonakis et al. 2000). In addition, serotonin increases $I_h$ in neurons in CA1 hippocampal pyramidal neurons (Gasparini and DiFrancesco 1999) and in neurons in the thalamus (McCormick and Pape 1990), nucleus prepositus hypoglossi (Bobker and Williams 1989) and dorsal root ganglion (Cardenas et al. 1999); these effects of serotonin are mediated by an increase in cAMP. Conversely, receptors which inhibit adenylyl cyclase can reduce $I_h$ as demonstrated for opioids in nodose ganglion cells (Ingram and Williams 1994) and adenosine A1 receptor mediated effects in thalamic (Pape 1992) and laterodorsal tegmental neurons (Rainnie et al. 1994). Since 5-HT$_1$ receptors inhibit adenylyl cyclase, we considered the possibility that the serotonin-induced reduction in $I_h$ which we observed in dopaminergic VTA neurons might be mediated by a reduction in cAMP.

In the present study, intracellular application of cAMP from the recording pipette caused a small increase in the amplitude of $I_h$ in all dopaminergic VTA neurons tested, indicating that cAMP had diffused into the cell and was exerting a positive modulatory effect on $I_h$. This is consistent with
previous reports that increasing intracellular cAMP can increase \( I_h \) in dopaminergic neurons in the ventral tegmental area (Jiang et al. 1993) and substantia nigra (Cathala and Paupardin-Tritsch 1997). Four genes encoding \( I_h \) channels (HCN1-4) recently have been cloned; all have a conserved cyclic nucleotide-binding domain, but the isoforms differ in the strength of their modulation by cAMP (Santoro and Tibbs 1999; Santoro et al. 2000). All 4 HCN genes are expressed in brain but show different regional localization (Moosmang et al. 1999; Robinson and Siegelbaum 2003). It remains to be determined which HCN isoforms are present in dopaminergic VTA neurons and their sensitivity to modulation by cAMP relative to neurons in other brain areas.

If the serotonin-induced reduction in \( I_h \) which we observed in dopaminergic VTA neurons was due to a decrease in cAMP, then increasing intracellular cAMP would be expected to prevent or significantly attenuate this inhibitory effect. Serotonin still reduced \( I_h \) amplitude after intracellular administration of cAMP from the recording pipette and after bath application with the membrane permeable cAMP analogue dibutyryl-cAMP. The effect of serotonin on \( I_h \) in the presence of internal cAMP or external dibutyryl-cAMP was not significantly different from the effect of serotonin alone. These data suggest that the reduction in \( I_h \) amplitude in dopaminergic VTA neurons is not consistent with inhibition of adenylyl cyclase.

The results of the present study suggest that the serotonergic reduction of \( I_h \) in dopaminergic VTA neurons is mediated by protein kinase C. Specifically, serotonergic inhibition of \( I_h \) was reduced by the non-specific kinase inhibitor H-7 and the selective protein kinase C inhibitor chelerythrine. Furthermore, the phorbol ester PDA mimicked and occluded the serotonin-induced reduction in \( I_h \). In addition to the reduction in \( I_h \) amplitude, serotonin also caused a negative shift in the voltage-dependence of activation of \( I_h \) in dopaminergic VTA neurons. Interestingly, protein kinase C also has been implicated in the reduction of \( I_h \) by neurotensin in dopamine neurons in the substantia nigra.
pars compacta (Cathala and Paupardin-Tritsch 1997). While the size of the decrease in I_h amplitude by neurotensin was similar to the serotonin-induced reduction in our study, neurotensin did not significantly change the voltage-dependence of activation of I_h (Cathala and Paupardin-Tritsch 1997). In view of the fact that there are numerous isoforms of PKC (Way et al. 2000), it is possible that neurotensin activates a different PKC isoform than serotonin. This might result in phosphorylation of different sites on the h-channel, or phosphorylation of a different subset of h-channels by the two neurotransmitters, leading to differences in their modulation of I_h.

Since I_h activates at membrane potentials negative to -70 mV in dopaminergic VTA neurons, it may not contribute much to their spontaneous firing rate (but see (Seutin et al. 2001)). Interestingly, we have presented data in the present paper that reduction of I_h can increase the inhibitory effect of dopamine on the spontaneous firing rate of dopaminergic VTA neurons. We have previously reported that serotonin, acting through 5-HT_2 receptors, also potentiates the inhibitory action of dopamine (Brodie and Bunney 1996). As even low concentrations of dopamine (e.g., 1 μM) were potentiated by ZD7288, the effect of I_h blockade on dopamine inhibition probably did not require reducing the whole cell membrane potential to -70 mV, since the cells were still spontaneously active. While further experiments will be necessary to uncover the precise mechanism of this action, it may be that the opening of the G-protein coupled inwardly-rectifying potassium channels (GIRKs) by the action of dopamine on D_2 receptors (Kim et al. 1995; Lacey et al. 1987) produces local membrane potentials negative to -70mV, which causes the opening of h-channels in the vicinity of those GIRK channels. The inhibitory dopamine current would be shunted by the reduced membrane resistance due to the open h-channels, and the effect of the inhibitory dopamine current on the whole cell would be reduced. When I_h is reduced by serotonin or ZD7288, the action of dopamine would
not be attenuated by the opening of nearby h-channels, and there would be relatively greater reduction of firing rate by dopamine.

The neuromodulatory role of serotonin may also extend to excitatory burst firing of dopaminergic neurons. Selective blockade of I_h with ZD7288 has been shown to decrease spontaneous firing of midbrain dopamine neurons (Seutin et al. 2001), and as hypothesized by those authors, I_h may be more important when the membrane achieves more negative potentials, for example, during the afterhyperpolarization following action potential burst firing. Following a burst, the afterhyperpolarization can achieve potentials negative to -70 mM, and the duration of this afterhyperpolarization may be reduced by the opening of h-channels. Reduction of I_h by serotonin may prolong the post-burst afterhyperpolarization and reduce excitability. This more sustained decrease in neuronal excitability might reduce the response to subsequent excitatory stimulation and limit the frequency of bursting in these neurons. As burst firing may be induced by excitatory amino acid neurotransmission (Johnson et al. 1992), this action of serotonin could have a neuroprotective effect on mesencephalic dopamine neurons.
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FIGURE LEGENDS

FIGURE 1. Serotonin effect on voltage responses and reduction of I_h.

Voltage responses of a dopaminergic VTA neuron evoked by a series of hyperpolarizing current pulses in increments of 30 pA before (A) and in the presence (B) of 300 μM serotonin. The voltage response reached a peak followed by a depolarizing sag. Serotonin (300 μM) decreased the amplitude of the sag indicating inhibition of I_h. C, Voltage/Current (V/I) curves in the absence (open symbols) and presence (solid symbols) of 300 μM serotonin. The peak (pk) and steady-state (ss) amplitude of voltage responses (from panels A and B) are plotted as a function of the current amplitude used to evoke them. Compared to control, 300 μM serotonin reduced the steady-state voltage responses, but had only a small effect on peak voltage responses. D, The difference between the peak and steady-state voltage (Δ Voltage) was plotted as a function of the peak voltage attained, for the response to each current pulse (same neuron as in panels A-C). Note that the voltage difference between peak and steady-state increased progressively at peak voltages more negative than –70 mV, which is the voltage range for activation of I_h. Serotonin (300 μM, solid circles) decreased the voltage difference between peak and steady-state as compared to control (open circles) indicating a reduction in I_h.
FIGURE 2. Reduction of $I_h$ by serotonin was concentration-dependent.

A, V/I curves similar to Fig. 1C show the peak and steady-state voltage responses evoked by a series of hyperpolarizing current pulses in control and in the presence of four different concentrations of serotonin in a single dopaminergic VTA neuron. Serotonin (5-HT, 50 to 500 μM) caused a concentration-dependent reduction in the steady-state voltage responses (Steady-State: symbols in legend) but had no effect on the peak responses (Peak: symbols in legend). B, The magnitude of $I_h$ in this neuron was determined from the difference between the peak and steady-state voltage responses and is plotted as in Fig. 1D. Serotonin (50 to 500 μM) caused a concentration-dependent decrease in the magnitude of $I_h$ at all potentials negative to −70 mV.

FIGURE 3. Serotonin-induced reduction of $I_h$ in a voltage-clamp recording.

A, Upper panel shows the hyperpolarizing voltage steps used to elicit the current responses shown in the lower panel. Note the time-dependent increase in inward current reflecting the activation of $I_h$. The instantaneous current (ins.) (arrow) was measured just after the decay of the capacitive transient and the steady-state current (ss.) (arrow) was measured near the end of the voltage command. B, Bath application of serotonin (500 μM) greatly reduced the amplitude of the steady-state current responses evoked with the same voltage protocol (same neuron as in A). C, Current/Voltage (I/V) curves showing the instantaneous (circles) and steady-state (triangles) currents as a function of the step voltage, in control (open symbols) and in the presence of 500 μM serotonin (filled symbols). Serotonin decreased the steady-state current at all voltage steps negative to −70 mV but had little or no effect on the instantaneous current. D, The difference between the instantaneous and the steady-state currents (amplitude of $I_h$) is plotted versus step voltage, in the absence (open circles) and presence (filled circles) of 500 μM serotonin.
FIGURE 4. Concentration-dependence of serotonin reduction of $I_h$ under voltage clamp conditions.
A. $I/V$ curve showing the instantaneous (open symbols) and steady-state (filled symbols) currents plotted as a function of the hyperpolarizing voltage step for a typical dopaminergic VTA neuron. Current responses were measured in control and after bath applications of three different concentrations of serotonin (5-HT, 100, 300 and 500 μM) to the same neuron (symbols on legend). Serotonin (100 μM to 500 μM) decreased the steady-state currents in a concentration-dependent manner, but did not affect the instantaneous currents.
B. $I_h$, the difference between the instantaneous and steady-state currents, was inhibited in a concentration-dependent manner by serotonin (100 μM to 500 μM); same neuron as in A. C. Pooled concentration-response curve for the effect of serotonin on $I_h$ amplitude in voltage clamp experiments. The amplitude of $I_h$ at a command voltage of -120 mV was measured by subtracting the instantaneous current from the peak current and expressed as percent change from control $I_h$ amplitude (see Methods). One or more concentrations of serotonin (30, 100 or 300 μM) was applied to 18 dopaminergic VTA neurons and the mean change in $I_h$ was plotted as a function of serotonin concentration (± S.E.M.).

FIGURE 5. The reduction in $I_h$ by serotonin was reversible upon washout.
Current/Voltage curve of $I_h$ amplitude (similar to Fig.3D) in a dopaminergic VTA neuron. Serotonin (300 μM, 5-HT, filled triangles) caused a large decrease in $I_h$ over control (open circles) which was fully reversible after about one hour of washout (wash, filled squares).
FIGURE 6. The 5-HT₂ agonist, α-methylserotonin (α-Me5-HT), reduces Iₜₙ.

A, Current/Voltage curve of Iₜₙ amplitude in a dopaminergic VTA neuron. α-Me5-HT (25 μM, filled circles) caused a clear decrease in Iₜₙ as compared to control (open circles). In 8 dopaminergic VTA neurons tested, α-Me5-HT (25 μM) significantly reduced the mean Iₜₙ amplitude measured at -120 mV from -281 ± 26 pA in control, to -229 ± 29 pA in α-Me5-HT (paired t-test, p < 0.001). B, The reduction of Iₜₙ by α-Me5-HT was reversible upon washout in all 3 dopaminergic VTA neurons tested. Specifically, α-Me5-HT significantly decreased the mean Iₜₙ amplitude measured at -120 mV from -258 ± 5 pA in control to -197 ± 6 pA in α-Me5-HT, and then fully reversed with washout to -251 ± 16 pA (one way repeated measures ANOVA, p = 0.014, Student-Newman-Keuls post hoc tests, p<0.05). The asterisk indicates a significant difference both from control and from wash. There was no difference between control and wash (p>0.05).
**FIGURE 7. Serotonin causes a negative shift in the voltage-dependence of activation of I_h.**

I_h currents measured in 10 dopaminergic VTA neurons were converted to conductance (G_h) with the equation

\[ G_h = \frac{I_h}{(E - E_{rev})} \]

where E is the voltage step used to evoke the current and E_{rev} is the reversal potential of I_h. The values for E_{rev}, which were calculated by tail current analysis, were -39 mV in control and -42 mV in 300 μM serotonin (see Methods section for details). The conductance (G_h) for each cell was normalized (G_h/G_{max}), where G_{max} was taken to be the value of G_h at -130 mV. The mean normalized conductance (G_h/G_{max} ± SEM) in the absence (open circles) and presence (closed circles) of serotonin (300 μM) is plotted as a function of voltage. Serotonin shifted this curve in the negative direction indicating a negative shift in the voltage-dependence for activation of G_h. This shift was statistically significant (n=10, two-way ANOVA, p=0.001). The half-activation voltage (V_{1/2}) determined from the Boltzmann equation fit to the data was -88 mV in control and -95 mV in 300 μM serotonin, a negative shift of about 7 mV. The smooth curves show the Boltzmann fit to the data points.
FIGURE 8.

Effect of cAMP agonists and protein kinase inhibitors on the serotonin-induced reduction of $I_h$. Bars indicate the mean (+ S.E.M.) percent decrease in $I_h$ measured at -120 mV. Serotonin alone (5-HT, 300 μM) reduced the amplitude of $I_h$ (-39.1 ± 5.6%, n=12). Serotonin (300 μM) also reduced $I_h$ amplitude in experiments in which 1 mM cAMP was included in the recording pipette (+cAMP, -32.5 ± 7.1%, n=5). $I_h$ was also reduced when 300 μM serotonin was applied in the presence of 500 μM dibutyryl-cAMP (+dBcAMP, -27.5 ± 4.7%, n = 3), a membrane permeable cAMP analogue. When the protein kinase inhibitor H-7 (75 μM) was included in the recording pipette, 300 μM serotonin caused only a very small mean percent decrease in $I_h$ amplitude (+H-7, -4.4 ± 11.0%, n=4). Likewise, when the protein kinase C inhibitor chelerythrine (25 μM) was included in the recording pipette, the reduction in $I_h$ amplitude by 300 μM serotonin (+Chelery, -16.2 ± 3.8%, n=7) was much smaller than the effect of serotonin alone. There was a significant difference between the drug conditions (one way ANOVA, p = 0.009) and Dunnett’s post hoc comparisons demonstrated a significant difference between the control effect of serotonin alone and the effect of serotonin in the presence of H-7 and chelerythrine (p < 0.05), but the effect of serotonin in the presence of internal cAMP or external dibutyryl-cAMP was not significantly different from serotonin alone (p>0.05).
FIGURE 9. The protein kinase C activator phorbol 12, 13 diacetate (PDA) reduces $I_h$.

A, Current/Voltage (I/V) curves showing the instantaneous (circles) and steady-state (triangles) currents as a function of the step voltage in control (open symbols) and in the presence of 2 μM PDA (filled symbols). DMSO (0.1%) was used as a vehicle and was present both in control and in the PDA-containing media. Like the effect of serotonin shown in Fig. 3C, PDA decreased the steady-state current but had little or no effect on the instantaneous current. B, The difference between the instantaneous and the steady-state currents ($I_h$ amplitude) is plotted versus the step voltage in the absence (open circles) and presence (filled circles) of 2 μM PDA. Note the large reduction in $I_h$ amplitude in the presence of PDA which closely resembles the effect of serotonin shown in Fig. 3D.

FIGURE 10. PDA mimics and occludes the serotonin-induced reduction in $I_h$.

Bars indicate the mean (+ S.E.M.) percent decrease in $I_h$ measured at -120 mV. The presence of DMSO vehicle alone, did not change $I_h$ amplitude (-1.7 ± 5.0%, n=7). In the presence of DMSO, 300 μM serotonin (5-HT) reduced the amplitude of $I_h$ (-35.3 ± 9.1%, n=4). The addition of phorbol 12,13 diacetate (PDA, 2 μM) in DMSO vehicle also reduced $I_h$ (-44.9 ± 7.8 %, n=3). In the presence of PDA (2 μM) and serotonin (300 μM), the amplitude of $I_h$ was decreased (-51.5 ± 20.0 %, n=3), however, this effect was much less than the sum of the effects of serotonin and PDA when tested alone. Asterisks indicate a significant difference from DMSO vehicle alone. (One way ANOVA, p=0.006; Student-Newman Keuls post hoc tests p < 0.02). The decreases in $I_h$ measured in 5-HT, PDA and 5-HT plus PDA were not significantly different from each other (p>0.05).
**Figure 11. Blockade of h-current with ZD7288 increases the inhibitory response to dopamine.**

Firing rate of dopaminergic VTA neurons was measured with extracellular recording techniques (see Methods).

A. Ratemeter recording of firing rate in the absence of ZD7288. Each vertical line represents the average firing rate over a 5 sec interval, and the horizontal bars indicate the duration of application of dopamine (1, 2, 5 or 10 μM as labeled). Percent change in firing rate produced by dopamine was assessed by comparing the firing rate over a one-minute interval at the peak dopamine effect to the firing rate over a one-minute interval immediately prior to dopamine administration (see Methods). In this cell, 5 μM dopamine produced a -7.7% decrease in firing, and 10 μM produced a -14.6% decrease in the firing rate.

B. Ratemeter recording of firing rate of the same cell as in part A in the presence of 30μM ZD7288. In the presence of ZD7288, 5 μM dopamine produced a -31.1% decrease in firing, and 10 μM produced a -64.8% decrease in the firing rate. C. Pooled concentration-response curve showing the mean change in firing rate produced by dopamine plotted as a function of dopamine concentration in the absence (●) and presence (■) of 30 μM ZD7288 (n=14). The effect of dopamine in the presence of ZD7288 was significantly greater than the effect of dopamine prior to administration of ZD7288 (two-way ANOVA, p< 0.001).
Table 1. *Current clamp data showing the effect of different concentrations of serotonin on \( I_h \) measured in 21 dopaminergic VTA neurons.*

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</table>

Each neuron was tested with from one to four different concentrations of serotonin. When multiple concentrations were tested on the same neuron, the order of administration was from lowest to highest and each concentration was applied for 10-15 min before increasing the concentration. The number of cells showing each type of response is listed with the percent response of all cells tested with the same concentration given in parentheses.
Table 2. Voltage clamp data showing the effect of different concentrations of serotonin on $I_h$ measured in 40 dopaminergic VTA neurons.

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</tr>
<tr>
<td>300</td>
<td>17 (94.4%)</td>
<td>0 (0%)</td>
<td>1 (5.6%)</td>
<td>18</td>
</tr>
<tr>
<td>500</td>
<td>2 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>2</td>
</tr>
</tbody>
</table>

Each neuron was tested with from one to four different concentrations of serotonin. When multiple concentrations were tested on the same neuron, the order of administration was from lowest to highest and each concentration was applied for 10-15 min before increasing the concentration. The number of cells showing each type of response is listed with the percent response of all cells tested with the same concentration given in parentheses.
Figure 1

A
Control

B
300 μM Serotonin

C

D

Current (nA)

Δ Voltage (mV)

Voltage (mV)

Voltage (mV)

Time (ms)

Time (ms)

-330 pA

-120
Figure 6

A

Voltage (mV)

-140 -120 -100 -80 -60 0

\[ h(I) \text{ (pA)} \]

-320 -240 -160 -80

- - - - -

Control

\( \alpha \)-Me-5-HT (25 \( \mu \text{M} \))

B

\[ I_h \text{ amplitude (pA)} \]

Control

\( \alpha \)-Me5-HT

Wash

*
Figure 7

- control $V_{m0} = -88$ mV
- serotonin $V_{m0} = -95$ mV
Figure 8

[Bar chart showing the decrease in Ih (%) for different treatments.]

- 5-HT
- cAMP
- dBcAMP
- H-7
- Chelery
Figure 9

(A) Voltage (mV) vs. Current (pA)
- DMSO control - ins
- DMSO control - ss
- 2 μM PDA - ins
- 2 μM PDA - ss

(B) Voltage (mV) vs. Current (pA)
- DMSO control
- 2 μM PDA
Figure 10
Figure 11

(A) Control

(B) ZD7288 30 μM

(C) Change in firing rate (%) vs. Dopamine concentration (μM)

- Control
- ZD7288 30 μM