

Direct and decarboxylation-dependent effects of neurotransmitter precursors on firing of isolated monoaminergic neurons

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Abstract To elucidate mechanisms that underlie the profound physiological effects of the monoamine precursors 5-hydroxy-L-tryptophan (5-HTP) and L-3,4-dihydroxyphenylalanine (L-DOPA), we examined their action on single monoaminergic neurons isolated from the ganglia of the gastropod snail *Lymnaea stagnalis*. In isolated serotonergic PeA motoneurons, 5-HTP produced excitation. The effect was mimicked by serotonin at 0.5–1 μ M, masked by pretreatment with serotonin at higher concentrations, and abolished by the inhibitor of aromatic amino acid decarboxylase (AAAD) m-hydroxybenzylhydrazine (NSD-1015), the inhibitor of the vesicular monoamine transporter reserpine or the serotonin receptor antagonist mianserin. Exposure of the dopaminergic interneurons RPeD1 to L-DOPA caused a biphasic effect composed of a depolarization followed by a hyperpolarization. AAAD inactivation with NSD-1015, as well as the blockade of dopamine receptors with sulpiride, resulted in the enhancement of the excitatory effect, and the abolition of the inhibitory effect. Dopamine caused hyperpolarization and masked the inhibitory phase of L-DOPA action. The results show that precursors affect the rate of firing of isolated monoaminergic neurons and that their effect is completely or partially mediated by the enhanced synthesis of the respective neurotransmitter, followed by extrasynaptic release of the latter and activation of extrasynaptic autoreceptors.

Keywords L-DOPA · 5-hydroxytryptophan · Extrasynaptic release of neurotransmitter · Isolated neuron · *Lymnaea stagnalis* · Dopaminergic neuron · Serotonergic neuron

Abbreviations

5-HTP	5-hydroxy-L-tryptophan
L-DOPA	L-3,4-dihydroxyphenylalanine
AAAD	Aromatic amino acid decarboxylase
NSD-1015	m-hydroxybenzylhydrazine

Introduction

The immediate metabolic precursor of dopamine L-3,4-dihydroxyphenylalanine (L-DOPA) and the precursor of serotonin 5-hydroxy-L-tryptophan (5-HTP) are widely used as tools to enhance the activity of the dopamine and serotonin neurotransmitter systems. These two precursors produce dramatic changes in physiological and behavioral events (Marinesco et al. 2004b; Sakharov 1991; Ureshi et al. 2002). Each precursor also has clinical significance. L-DOPA is used to ameliorate motor disturbances in Parkinson's disease (Heifi and Melamed 1980) and may be involved in the poisoning effect of ecstasy (Breier et al. 2006), while 5-HTP is known to play a role in the pathophysiological mechanism of poisoning caused by antidepressants (Birdsall 1998; Byerley et al. 1987; Martin 1996).

Understanding the cellular mechanisms of L-DOPA and 5-HTP action is important for answering questions about how neurons control their own firing rate, especially with regard to how the level of neurotransmitter synthesis impacts the cell's firing rate. Many of the underlying cellular mechanisms remain, however, unknown.

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Treatment with a precursor is known to increase intracellular intravesicular levels of the associated transmitter (Colliver et al. 2000; Fickbohm et al. 2005; Pothos et al. 1996, 1998; Roseberry et al. 2007). However, elevated intravesicular levels of dopamine or serotonin alone cannot account for the observed behavioral effects. Additional hypotheses have thus been proposed. They include the suggestion that the precursors act directly upon the neuronal membrane (Poon 1980; Touret et al. 1991), and the hypothesis that feedback mechanisms may operate to adjust the electrical activity of the neuron to levels that are appropriate to the perceived level of neurotransmitter synthesis (Kabotyanski et al. 1991). Conclusive evidence has not yet been obtained because necessary experiments that use isolated monoaminergic neurons have not been performed.

We have exploited advantageous features of the *Lymnaea stagnalis* preparation to test the above-mentioned hypotheses using isolated monoaminergic neurons, the dopaminergic interneurons RPeD1 (Haydon and Winlow 1981; Magoski et al. 1995), and the serotonergic cilia motoneurons PeA2 and PeA8 (Kemenes et al. 1989; Syed and Winlow 1989). Our study attempts to answer the following questions: (1) Can isolated neurons respond to precursors by changing their firing rates or are the cellular responses caused by L-DOPA and 5-HTP only network events? (2) Within a single neuron, does the precursor act directly on the electrical activity of the neuron or is the precursor first converted into the neurotransmitter? We used a previously developed method for neuron isolation that allows us to continuously record cell activity during isolation and then perform experiments during first hours after isolation. To prevent conversion of precursors into their respective neurotransmitters, the commonly used aromatic amino acid decarboxylase (AAAD) inhibitor 3-hydroxybenzylhydrazine dihydrochloride (NSD-1015) was applied. This inhibitor prevents precursor molecules from decarboxylation also in molluscs (Fickbohm et al. 2005).

We report here results demonstrating that precursors affect the rate of firing in isolated monoaminergic neurons. We also show that both direct and metabolic effects may be involved in the polarizing action. We also provide data suggesting that, in the immediate environment of an isolated neuron, a varying concentration of the transmitter exists, which depends upon the amount of neurotransmitter synthesis and is capable of affecting firing rates.

Materials and methods

Animals

Mature specimens of *L. stagnalis* were taken from a breeding colony kept in dechlorinated tap water at room temperature

and fed on lettuce. Central ganglia were dissected from an animal anesthetized with injection of 0.1 mM MgCl₂. The central ganglia were placed into 2.5 mg/ml solutions of pronase E (Sigma) for 15 min, washed in a standard snail Ringer's solution (50 mM NaCl, 1.6 mM KCl, 4 mM CaCl₂, 8 mM MgCl₂, 10 mM Tris, pH 7.6) and pinned to sylgard in a 4 mm chamber. The connective tissue sheath was then removed from the pedal ganglia, and the preparation was kept undisturbed for three hours before single cell isolation and use in experiments.

Cell isolation and electrophysiology

Visual identification of the unpaired RPeD1 and paired PeA2/A8 neurons was performed based on their location, size, and coloration (Fig. 1). Other neurons were randomly taken from serotonergic Pedal A (PeA) clusters to assess whether the observed effects of drug administrations were common to different cluster members. The neuron that was selected for examination was impaled with a standard glass microelectrode (10–20 MΩ) filled with 3 M KCl. For neuron isolation, we utilized methods developed previously for terrestrial snails (Dyakonova 1991). Using the intracellular microelectrode as a pull (Fig. 2a, b, c), the neuron was gently pulled out-of the tissue until separation of the proximal neurite from the neuropile was achieved. Giant RPeD1 cell isolation was more complicated because of the cell size and, sometimes, when the cell tension was high, the second microelectrode was impaled into the distal end of the cell, and both electrodes were used as a pull. At the end of isolation, one of the two microelectrodes was removed. The electrical activity of the cell was monitored during isolation (Fig. 2d, start and end of neuron pulling are indicated with vertical lines). The cells, which demonstrated membrane injury, were not used in the experiments.

The isolated neuron was placed into a continuous stream of the Ringer's solution (0.75 ml/min). Prior to drug application, the cell was kept chemically unstimulated for at least 20 min until its background electrical activity became stable. A standard setup for microelectrode recording was used. The electrophysiological recordings were stored in computer files using a home-made program. In parallel, a chart recorder was used in some experiments.

Chemicals

L-3,4,-dihydroxyphenylalanine (L-DOPA), 3-hydroxytyramine (dopamine), 5-hydroxy-L-tryptophan (5-HTP), 5-hydroxytryptamine (serotonin), the inhibitor of aromatic amino acid decarboxylases m-hydroxybenzylhydrazine (NSD-1015), the inhibitor of vesicular monoamine transporters reserpine, the vertebrate D2 dopamine receptor antagonist sulpiride and the vertebrate 5HT2 serotonin receptor antagonist

Fig. 1 Positions of the investigated neurons at the dorsal surface of the paired pedal ganglia of *Lymnaea stagnalis*. PeA2, PeA8 and RPeD1 neurons (arrows). 18 superior pedal nerve, 20 medial pedal nerve, 24 cerebro-pedal connective, 25 pleuro-pedal connective, St statocyst. Modified from Slade et al. 1981

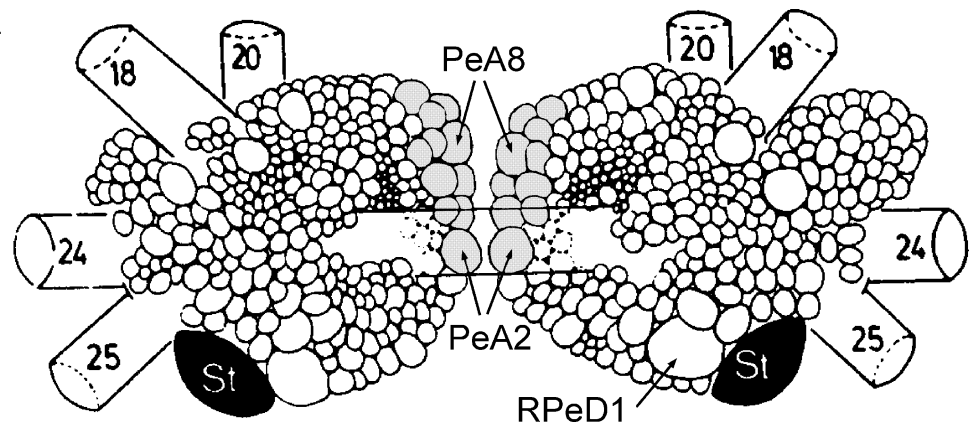
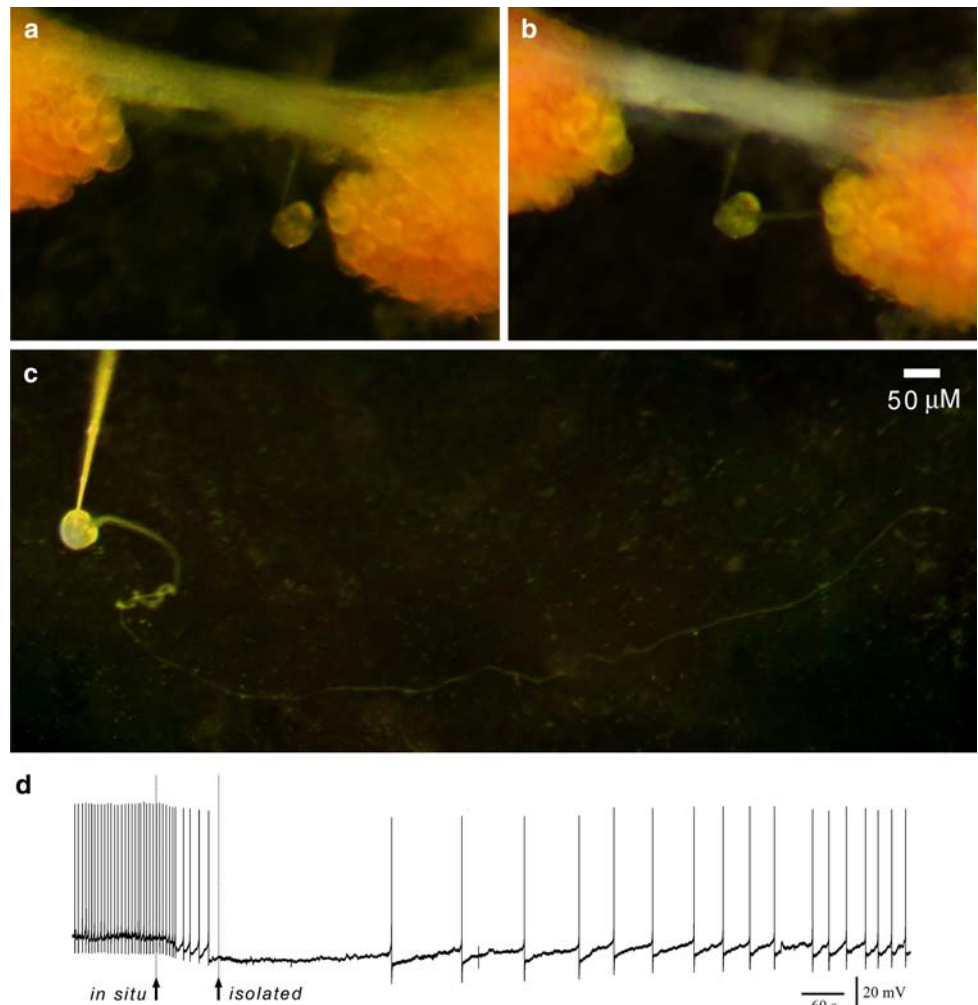


Fig. 2 Isolation of a neuron. **a** Start of isolation. **b** Semi-isolated cell with its neurites in the ganglion. **c** Completely isolated neuron. **d** Electrical activity of the cell; vertical lines indicate start and end of isolation (MP: -45 mV in situ, -53 mV at 10 min of isolation)



mianserin were all obtained from Sigma (Moscow, Russia) and were applied to the bath by switching superfusion inflow reservoirs. Preliminary experiments were performed to find effective dose ranges for these drugs. Pretreatment with a drug was performed for 20 min for NSD-1015 and reserpine and for 3–5 min for sulpiride, mianserin, dopamine, and serotonin prior to a 4–5 min administration of the transmitter precursor dissolved in Ringer’s solution. The

transmitter precursor solution also contained the pre-treatment chemical. Subsequent washout was performed for 10–15 min.

Data analysis

The significance of differences in spike frequency prior to and after drug administration was tested either by the paired

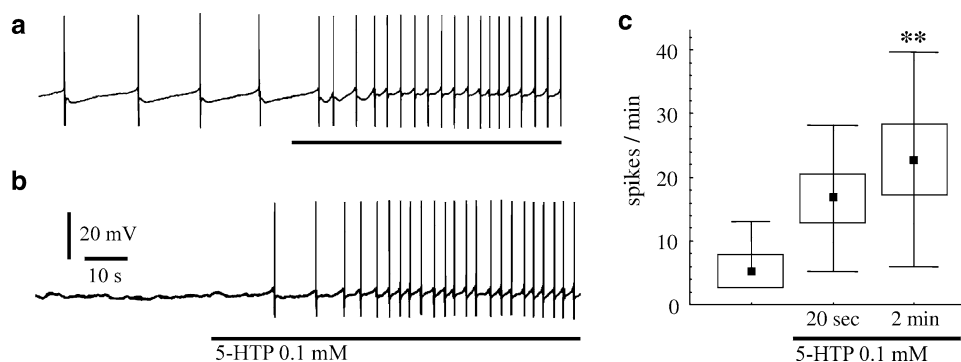


Fig. 3 Effect of 5-HTP on isolated serotonergic PeA neurons. **a, b** Bath application of 0.1 mM 5-HTP in Ringer's solution enhances firing rate in initially active (**a**) and initially silent (**b**) neurons. **c** Mean frequency

of spiking measured prior to, in 20 s and in 2 min of bath application of 0.1 mM 5-HTP. ** indicates $P < 0.01$ in comparison to the control (one-way repeated measures ANOVA with Tukey post hoc test)

Wilcoxon signed-rank test for dependent samples or by the nonparametric Kruskal-Wallis ANOVA test using the STATISTICA program (StatSoft Inc. 1993). One-way repeated measures ANOVA procedures followed by post hoc Tukey tests for multiple comparison were used when appropriate. All values are given as mean with standard error and level of significance. There was no significant difference between serotonergic PeA2 and PeA8 neurons in their initial membrane potential, spike frequency, and responses to drugs. For that reason, the data obtained from PeA2 and PeA8 were pooled.

Results

The serotonin precursor affects firing of serotonergic PeA neurons

5-HTP excites isolated serotonergic cells

Application of 5-HTP depolarized and activated the isolated PeA2 and PeA8 neurons ($n = 9$). Prior to 5-HTP application, the frequency of the discharge in initially active isolated neurons was 9 ± 3 spikes/min ($n = 5$) at a membrane potential of -55 ± 3 mV. This value increased to 20 ± 6 spikes/min after 1 min of 0.1 mM 5-HTP application ($z = 2$, $P < 0.05$, paired Wilcoxon signed-rank test for dependent samples) (Fig. 3a). In all initially silent isolated neurons (membrane potential -69 ± 4 mV, $n = 4$), this 5-HTP concentration produced depolarization followed by firing (13 ± 8 spikes/min after 1 min of drug application) (Fig. 3b). In Fig. 3c, the data from all nine cells (initially active and initially silent) are combined. The measurements were made at the 20–40 s and 2 min post 5-HTP administration time points. Electrical activity returned to the initial level after washing for 10–20 min (not illustrated).

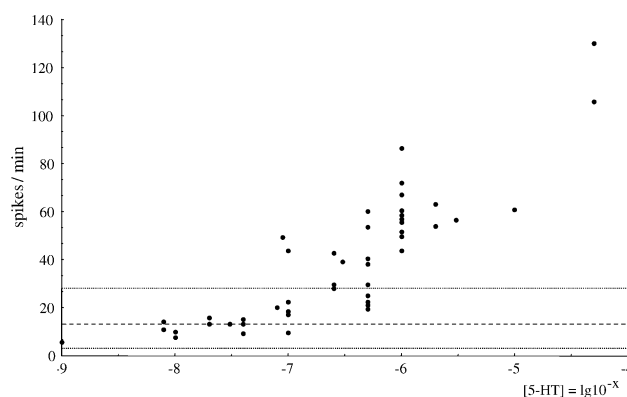


Fig. 4 Comparison of serotonin and 5-HTP effects on the electrical activity of isolated PeA neurons. Filled circles show the rate of firing (spikes/min) of individual isolated PeA neurons ($n = 36$) at various concentrations of serotonin. Dotted horizontal lines show mean with minimal and maximal values of spiking rate in 0.1 mM 5-HTP ($n = 10$ cells, data from experiments performed in parallel with serotonin application experiments)

In addition, effects of 5-HTP were tested on isolated serotonergic PeA neurons other than PeA2/8 ($n = 14$, membrane potential -62 ± 3 mV). Similar to the PeA2/8 cells, all cells responded to the serotonin precursor with excitation. The mean frequency before and 5 min after 0.1 mM 5-HTP application was 8 ± 1 and 20 ± 2 spikes/min, respectively ($z = 3.5$, $P < 0.001$, paired Wilcoxon signed-rank test for dependent samples).

Serotonin excites isolated PeA neurons

Serotonin at 0.01–10 μ M caused depolarization and increases in the firing rate of isolated serotonergic PeA neurons ($n = 45$ measurements, 36 cells). Figure 4 illustrates maximal values of spike frequency of individual PeA neurons in serotonin at various serotonin concentrations. Dotted horizontal lines show the mean with minimal and maximal values of spike rate in 0.1 mM 5-HTP ($n = 10$).

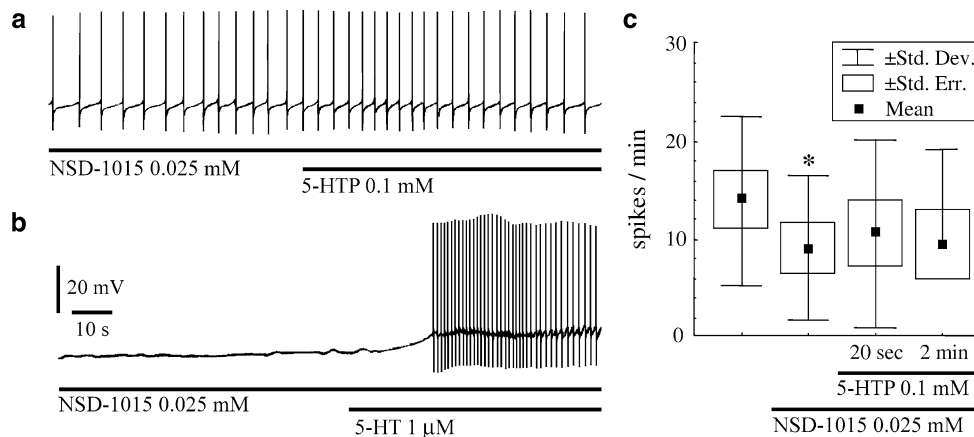


Fig. 5 Aromatic amino acid decarboxylase inactivation with m-hydroxybenzylhydrazine (NSD-1015) abolishes the excitatory effect of 5-HTP on isolated serotonergic PeA neurons. **a** No response to 5-HTP after pretreatment with 0.025 mM NSD-1015 for 20 min (46 mV). **b** Neuron, pretreated with NSD-1015, responds to 1 μ M serotonin. **c** Mean frequency of spiking measured in cells exposed to Ringer's solution, 20 min of bath application of NSD-1015, and 20 s and 2 min

of bath application of 0.1 mM 5-HTP in NSD-1015. Note, that NSD-1015 does not only abolish the excitatory effect of 5-HTP but does significantly decrease the rate of firing. Spike frequency is measured in spikes/min, all values are reported as mean with SEM and SD; * indicates $P < 0.05$ (one-way repeated measures ANOVA with Tukey post hoc test)

Inhibition of aromatic amino acid decarboxylation abolishes the excitatory action of 5-HTP

NSD-1015 (0.025 mM) produced slow hyperpolarization and decreased the electrical activity of isolated PeA2/A8 neurons ($P < 0.02$, one-way repeated measures ANOVA with Tukey post hoc test, Fig. 5c). In NSD-1015-treated neurons, 5-HTP caused no excitation (Fig. 8a) and the mean rate of firing remained almost constant after 5-HTP administration (Fig. 5c, $P = 0.9$). In contrast to 5-HTP, serotonin retained its excitatory action on NSD-1015-treated A2/A8 neurons (Fig. 5b).

The serotonin receptor antagonist mianserin decreases the excitatory effect of 5-HTP

The above effects of exogenous serotonin and NSD-1015 suggested that 5-HTP-induced excitation could be mediated by enhanced release of serotonin and subsequent activation of serotonin excitatory autoreceptors. To verify this hypothesis, we sought to determine the effect of 5-HTP on serotonergic neurons pretreated with a serotonin receptor antagonist.

We first tested the effectiveness of the vertebrate 5HT2 receptor antagonist mianserin on neurons studied here. We evaluated the effects of serotonin at two concentrations (0.5 and 1 μ M) on isolated PeA neurons pretreated with 10 μ M mianserin for 5–10 min. Figure 6a demonstrates that the effect of 0.5 μ M serotonin was completely blocked and that of 1 μ M was significantly decreased by mianserin. Mianserin itself produced slight hyperpolarization (from -59 ± 5 to -61 ± 4 mV, $z = 2$, $P < 0.05$, paired Wilcoxon

signed-rank test for dependent samples), and caused a firing rate decrease (Fig. 6b, c). Interestingly, the hyperpolarizing effect was seen even in silent PeA neurons. The effect was reversible as washing out the mianserin for 20–30 min resulted in cell depolarization and in the recovery of cell responsiveness to serotonin (10.4 ± 4 and $38 \pm$ spikes/min prior to and after 5HT administration).

The effects of 5-HTP were significantly diminished by mianserin (Fig. 6a, b, c). No difference was seen between rates of firing prior to and after 5-HTP application.

Exogenous serotonin at higher concentrations masks the effect of 5-HTP

The results of the above experiments with NSD-1015 and mianserin suggest that the effect of 5-HTP application is mediated by enhanced release of serotonin and subsequent activation of serotonin excitatory receptors. The excitatory effects of 0.1 mM 5-HTP were comparable to the effects of exogenous serotonin at concentrations below 1 μ M (see Fig. 4). To further demonstrate that 5-HTP effects were mediated by the release of endogenous serotonin, we tested the 0.1 mM 5-HTP action on PeA neurons pretreated with exogenous serotonin at different concentrations. We hypothesized that in the case that 5-HTP action is entirely mediated by endogenously released serotonin, 5-HTP would fail to exert an additional excitatory effect in the presence of exogenous serotonin at concentrations much higher than those resulting from 0.1 mM 5-HTP conversion.

5-HTP retained its excitatory action when the cell was incubated in 0.2 μ M serotonin (Fig. 7a) or 0.5 μ M serotonin (not shown) for 5 min. The firing frequency was

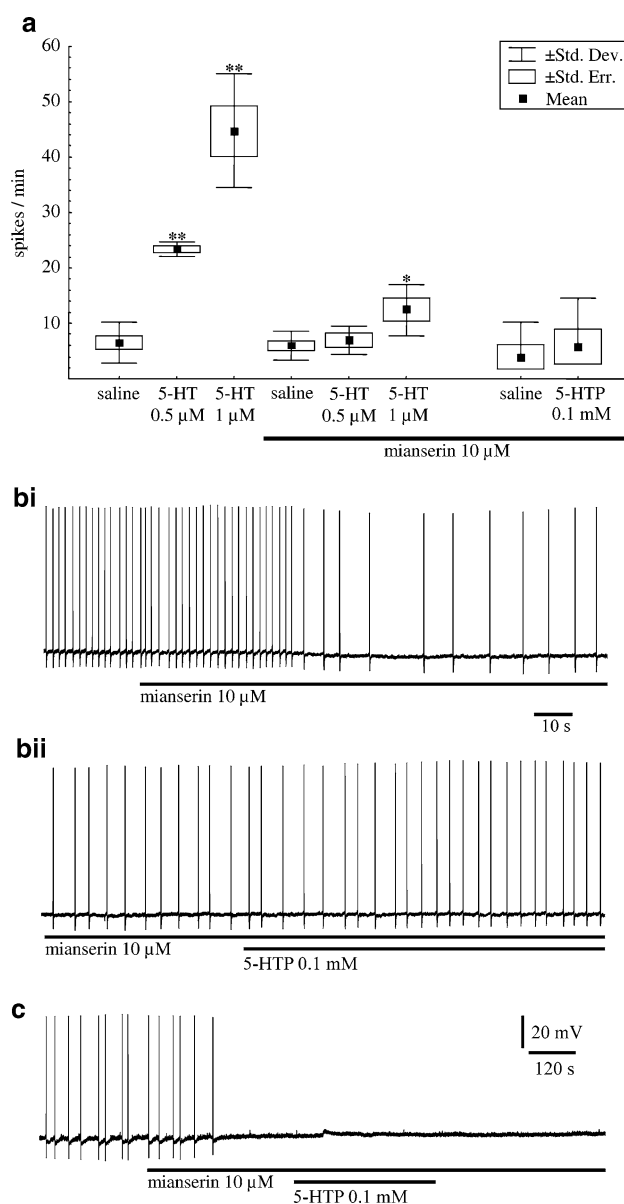


Fig. 6 Serotonin receptor antagonist mianserin abolishes the effect of serotonin and that of the serotonin precursor 5-HTP on firing of isolated PeA neurons. **a** Mean frequency of spikes measured in Ringer's solution only, 1 and 0.5 μ M serotonin solutions, a 0.01 mM mianserin solution, 0.5 and 1 μ M serotonin solutions with 0.01 mM mianserin, and a 0.1 mM 5-HTP solution with 0.01 mM mianserin. Spike frequency is measured in spikes/min, all values are reported as mean with SEM and SD; *, ** indicates $P < 0.05$ and 0.01, respectively. From left to right: $H(1,13) = 7.73$; $H(1,14) = 9$; $H(1,13) = 0.3$, $P = 0.53$; $H(1,14) = 6.1$; $H(1,16) = 0.3$, $P = 0.6$ in comparison to the related control (saline), H-Kruskal-Wallis ANOVA test results; **bi** Bath application of mianserin decreases the firing rate of individual PeA neuron (MP: -62 mV). **bii** Only weak effects can be seen after 5-HTP addition when compared with Fig. 3a. **c** Mianserin turns off firing of individual PeA cell (MP: -42 mV). Addition of 5-HTP does not result in reoccurrence of firing (compare with Fig. 3b)

increased 30% after the addition of 5-HTP to serotonin containing solution. Washing 5-HTP in serotonin containing solution resulted in a decrease in the firing

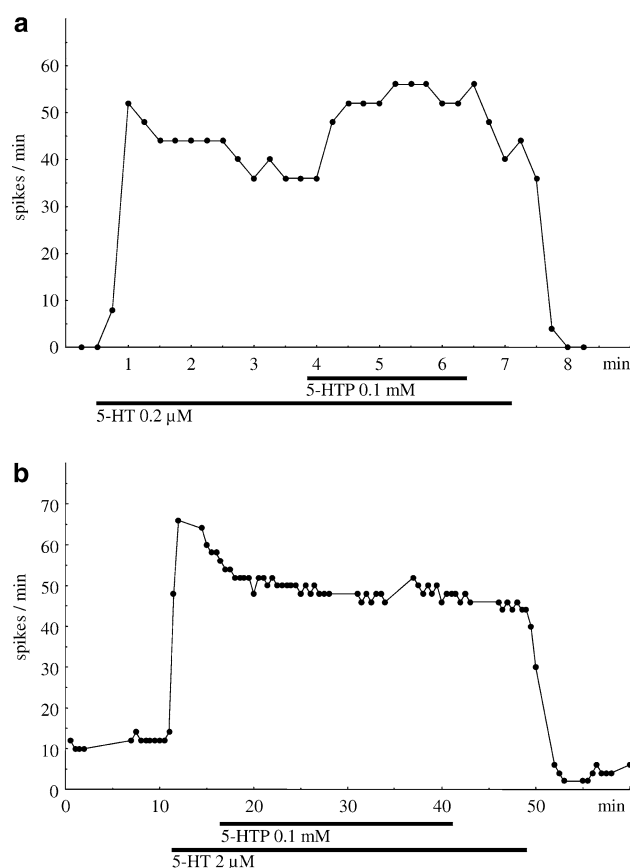


Fig. 7 Exogenous serotonin at higher concentrations masks the effect of 5-HTP. **a**, **b** Frequency of spikes of individual isolated PeA neurons. In **a**, an additional excitatory effect of 0.1 mM 5-HTP can be seen after pretreatment with 0.2 μ M serotonin. In **b**, no effect of 5-HTP can be seen after pretreatment with a two micromolar serotonin solution

frequency back to the initial levels. However, at 1.0, 2.0 (Fig. 7b) and 5.0 μ M concentrations, serotonin completely masked the effects of 0.1 mM 5-HTP in four of six experiments. In two of six cells, a light additional excitation was observed after 5-HTP application; however, it was not reversible and washing out 5-HTP did not result in the restoration of the initial level of spike frequency.

These results correspond well to those shown in Fig. 4. We observed additional effects of 0.1 mM 5-HTP in cells pretreated with serotonin at concentrations that were previously shown to cause weaker effects than the maximal effect of 0.1 mM 5-HTP. When serotonin was applied at higher concentrations, it would mask the effect of 5-HTP provided that the two substances acted on the same target. In this case, if the concentration of endogenously released serotonin, synthesized from 5-HTP, is much lower than the concentration of exogenous serotonin, then the effect of the former may not be large enough to cause changes in electrical activity.

Reserpine prevents the excitatory effect of 5-HTP

The above data strongly suggested that the effect of 5-HTP is mediated by enhanced release of endogenous serotonin. To investigate whether newly synthesized vesicular serotonin was involved in the mechanism of 5-HTP action, we tested the effect of 5-HTP on serotonergic neurons pretreated with reserpine, a known inhibitor of the vesicular monoamine transporter (e.g. Colliver et al. 2000).

First, we found that reserpine inhibited the previously reported (Kabotyanski et al. 1991) effect of 5-HTP on serotonergic PeA neurons in situ. In the control Ringer's solution, the firing frequency before and after 5-HTP application was significantly different (13 ± 4 and 22 ± 4 spikes/min, respectively, $n = 5$, $z = 2$, $P < 0.05$, paired Wilcoxon signed-rank test for dependent samples). The difference became insignificant in the presence of reserpine (16 ± 4 and 17 ± 4 spikes/min, respectively, $n = 8$, $z = 0.5$, $P = 0.6$, not illustrated).

There was no difference in the mean rate of firing prior to or after 5-HTP application ($n = 8$, $z = 0.8$, $P = 0.4$, paired Wilcoxon signed-rank test for dependent samples, Fig. 8) in isolated serotonergic neurons pretreated for 20–30 min with 0.1 mM reserpine. Of eight neurons, only two (25%) responded to 5-HTP with excitation. This is in contrast to all neurons that responded in the control. Four of the reserpine-treated neurons did not change their activity and membrane potential, and two responded with hyperpolarization to 5-HTP. Notably, reserpine also blocked the response to 5-HTP in silent neurons ($n = 2$). An excitatory effect of reserpine was observed in some isolated neurons. This could result from exocytotic release of serotonin induced by reserpine (Mundorf et al. 2000) or from some nonspecific effect of reserpine.

The data obtained from serotonergic PeA neurons, therefore, suggest that the excitatory effect of 5-HTP results from 5-HTP conversion to serotonin, enhanced vesicular release of serotonin, and activation of excitatory autoreceptors on the cell membrane.

Dopamine precursor affects firing of dopaminergic cells RPeD1

L-DOPA exerts a biphasic effect on isolated RPeD1 cells

Within 10–40 s of application, 0.1 mM L-DOPA caused excitation of the isolated RPeD1 cells (Fig. 9a). Five of thirteen isolated RPeD1 cells examined were initially silent at -71 ± 5 mV, and they all responded to L-DOPA administration with a depolarization that ranged from 3 to 6 mV. In two of five silent cells, depolarization produced action potentials. In eight RPeD1 cells that had initially been active at 24 ± 4 spikes/min and had a membrane potential

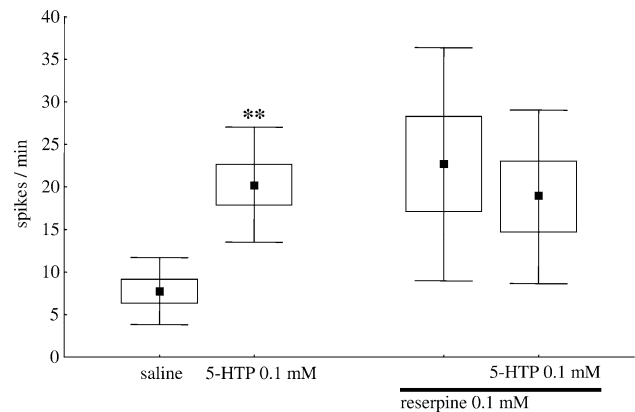


Fig. 8 An inhibitor of the vesicular monoamine transporter reserpine abolishes the effect of 5-HTP on activity of isolated serotonergic PeA neurons. Mean frequency of spikes of individual isolated PeA neurons prior to and after 2 min of bath application of 0.1 mM 5-HTP in Ringer's solution (*left*) and in 0.1 mM reserpine (*right*). Spike frequency is measured in spikes/min, action potentials are measured per minute, and all values are reported as mean with SEM and SD, ** indicates $P < 0.01$, the paired Wilcoxon test results are $z = 2.5$ with $P < 0.01$

of -63 ± 7 mV, treatment with L-DOPA resulted in a 2–4 mV depolarization and in an increase of firing frequency with the peak value of 35 ± 5 spikes/min after 20–40 s of drug application ($P = 0.01$, one-way repeated measures ANOVA with Tukey post hoc test). The excitatory action of L-DOPA never persisted longer than 2 min. It was followed by hyperpolarization (-7 ± 3 mV) (Fig. 9a), and the firing frequency decreased progressively toward its minimum value at 15 ± 4 spikes/min after 4 min. This value was significantly lower than that observed during the first minute of L-DOPA action ($P = 0.001$, one-way repeated measures ANOVA with Tukey post hoc test). In Fig. 9c, data from all 13 cells (initially active and initially silent) are combined. The firing frequency is evaluated 20–40 s and 2 min after L-DOPA administration.

The use of electrodes filled with 3 M KCl could result in a reversal of the chloride equilibrium potential which in turn could induce a depolarization instead of a physiological hyperpolarization. To rule out this possibility, we performed an additional series of experiments ($n = 10$) with RPeD1 cell in situ using electrodes filled with potassium acetate. In this series, L-DOPA administration caused typical biphasic effects (depolarization, then hyperpolarization) followed by activation of previously described respiratory motor program (Syed and Winlow 1991).

To test the possibility that some effects resulted from unspecific action caused by L-DOPA destruction, we compared the effect of the L-DOPA solutions kept at room temperature for a few seconds ($n = 5$), 30 min ($n = 3$), and 1 h ($n = 4$). Typical biphasic effects were observed in all cases. In addition, we tested for the effects

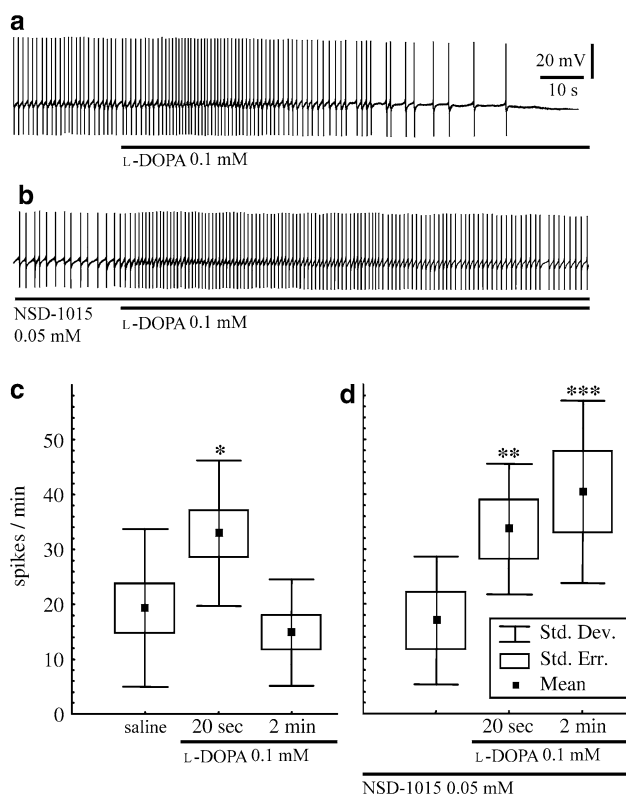


Fig. 9 Biphasic effect of L-DOPA on isolated dopaminergic cell RPeD1 and elimination of the inhibitory phase by the aromatic amino acid decarboxylase (AAAD) inhibitor NSD-1015. **a, c** In Ringer's solution, excitation followed by hyperpolarization can be seen after L-DOPA administration. **b, d** In NSD-1015-containing Ringer, only depolarization and increases in the firing rate can be seen after L-DOPA administration. In **c** and **d**, the activity of isolated dopaminergic neurons RPeD1 was measured prior to, at 20 s and at 2 min after bath application of 0.1 mM L-DOPA. Spike frequency is measured in spikes/min and all values are reported as mean with SEM and SD; *, ** and *** indicates $P < 0.05$, $P < 0.01$ and 0.001, respectively (one-way repeated measures ANOVA with Tukey post hoc test). Initial level of MP in **a, b** is -53 mV

of changes in the pH of 0.1 mM L-DOPA 4 h after the solution had been prepared. The changes in pH value were around 0.04 and were not large enough to produce any experimental effect.

Dopamine hyperpolarizes RPeD1

Dopamine at concentrations of 0.1 μ M ($n = 4$), 1 μ M ($n = 4$), 10 μ M ($n = 2$) and 100 μ M ($n = 7$) produced sustained hyperpolarization (Figs. 10, 11a, d), and in 15 of 17 cases, completely inhibited firing. Dopamine at 0.01 μ M ($n = 4$) had no effect on RPeD1 in two cases. In another two experiments, the same concentration produced very weak hyperpolarization. The hyperpolarizing effect of 0.1 mM L-DOPA was comparable with that of 0.1–1 μ M dopamine (Fig. 10).

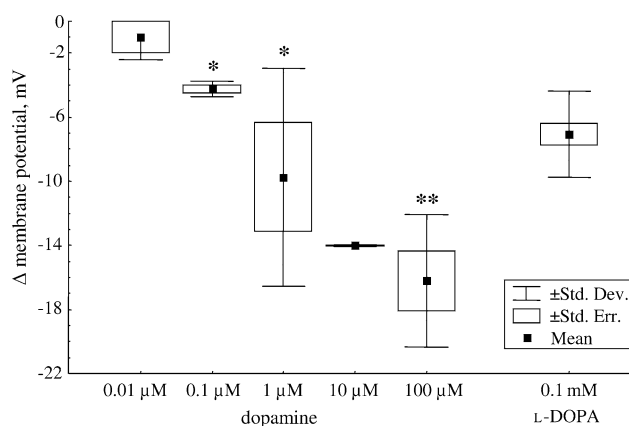


Fig. 10 Hyperpolarizing effects of exogenous dopamine and L-DOPA on isolated RPeD1 neurons. Mean delta membrane potential with SEM and SD at various concentrations of dopamine and 0.1 mM L-DOPA. *, ** indicate $P < 0.05$ and 0.01, respectively ($H = 6.4$; 6.0; 7.8 from left to right, Kruskal-Wallis ANOVA test results)

Inhibition of aromatic amino acid decarboxylation potentiates the excitatory phase of L-DOPA action, and abolishes the inhibitory one

In RPeD1 neurons pretreated with 0.05 mM NSD-1015, excitation was only observed following exposure to 0.1 mM L-DOPA (Fig. 9b). Figure 9d demonstrates that the mean initial rate of firing of RPeD1 cells in the NSD-1015 containing saline ($n = 5$) was significantly increased within 20 s of L-DOPA application and reached even higher values after 2 min. There was no significant difference between values measured after 1 versus 2 min of L-DOPA action. Washing L-DOPA out with basic Ringer's containing NSD-1015 returned the firing frequency to 18 ± 8 spikes/min within 10 min (not illustrated).

The D2 dopamine receptor antagonist sulpiride abolishes the inhibitory phase of L-DOPA action

The effects of exogenous dopamine and NSD-1015 suggest that the second phase of L-DOPA's action was mediated by endogenously synthesized dopamine. We next sought to determine whether the dopamine receptor antagonist abolished the inhibitory effect of L-DOPA. We tested the effect of L-DOPA on isolated RPeD1 cells pretreated with the D2 receptor antagonist sulpiride, which had been shown to abolish dopamine effects in *Lymnaea* (Spencer et al. 2000).

Sulpiride at 0.1 mM produced depolarization (2–3 mV) and significantly increased the firing rate of isolated RPeD1 cells (Fig. 11ci, ii; $z = 2.2$; $P < 0.03$, paired Wilcoxon signed-rank test for dependent samples). Interestingly, it caused a 3 mV depolarization even in initially silent

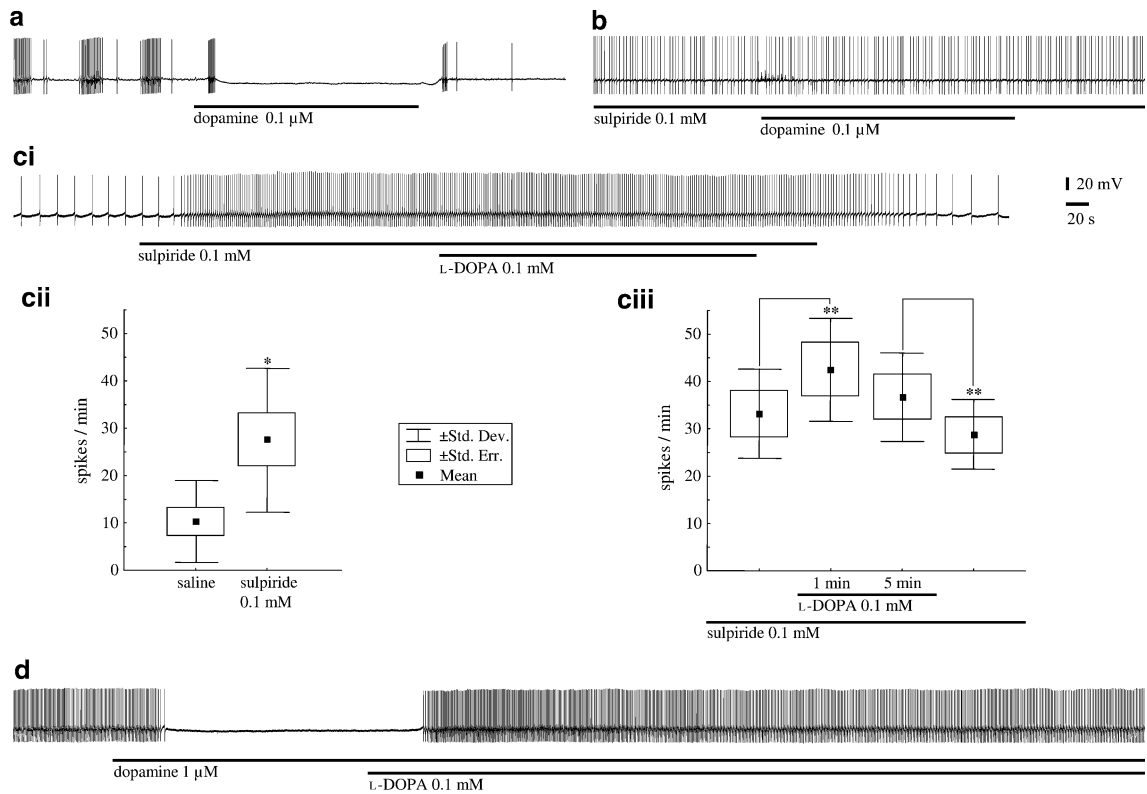


Fig. 11 Dopamine antagonist sulpiride and exogenous dopamine abolish the inhibitory phase of L-DOPA action. **a**, **b** 0.1 mM sulpiride blocks hyperpolarizing action of 0.1 μ M dopamine [MP: -45 mV (**a**), -43 mV (**b**)]. **c** Increases in the mean spike rate of isolated RPeD1 neurons caused by 0.1 mM sulpiride application and by application of a mixture of 0.1 mM L-DOPA with 0.1 mM sulpiride causes additional excitatory action and both effects are washable (MP: -69 mV). **ci** 0.1 mM sulpiride depolarizes isolated RPeD1, 0.1 mM L-DOPA in 0.1 mM sulpiride causes additional excitatory action and both effects are washable (MP: -69 mV). **cii** The RPeD1 activity measured prior to and after 3 min of treatment with 0.1 mM sulpiride; Spike frequency is measured in spikes/min and all values are reported as mean with SEM and

SD, * indicates $P < 0.05$, see other statistics in the “Results”. **ciii** The RPeD1 activity in 0.1 mM sulpiride prior to, after 1 and 5 min of 0.1 mM L-DOPA application and after washing L-DOPA in sulpiride for 5 min. Spike frequency is measured in spikes/min, all values are reported as mean with SEM and SD; the following comparisons are illustrated: L-DOPA in sulpiride 1 min versus sulpiride; washing L-DOPA in sulpiride versus L-DOPA in sulpiride 5 min, * and ** indicate $P < 0.05$ and 0.01 , respectively (one-way repeated measures ANOVA with Tukey post hoc test). **d** Exogenous dopamine (1 μ M) turns spiking off in isolated RPeD1 neurons while the addition of 0.1 mM L-DOPA causes sustained excitation (MP: -63 mV)

neurons. In agreement with earlier reports (Spencer et al. 2000), the drug had a prominent dopamine antagonizing effect and completely blocked the effects of exogenously applied 0.1 and 1.0 μ M dopamine solutions (Fig. 11a, b). The depolarizing effect of the antagonist may, therefore, suggest that resting dopamine release is responsible for tonic cell inhibition.

L-DOPA caused a 4–7 mV depolarization in RPeD1 kept in 0.1 mM sulpiride for 2–3 min ($n = 6$, Fig. 11ci, iii). After the first minute of L-DOPA action, the frequency of spikes significantly increased (Fig. 11ciii). There was no decrease after the second minute of L-DOPA treatment. Although in the following two minutes the electrical activity declined, it did not reach a level below the initial one measured in sulpiride ($P > 0.5$, one-way repeated measures ANOVA with Tukey post hoc test). A change in the applied solution to sulpiride alone significantly decreased the activity from 37 ± 5 to 29 ± 4 spikes/min (Fig. 11ci, iii).

Exogenous dopamine masks the inhibitory effect of L-DOPA

Sulpiride effectiveness in antagonizing the inhibitory phase of L-DOPA action suggests that the release of endogenous dopamine and subsequent activation of the inhibitory dopamine receptors at the cell membrane might underlie the inhibitory action of L-DOPA. We hypothesized that in the presence of exogenous dopamine at certain concentrations, L-DOPA would fail to exert additional inhibitory effects on the RPeD1 cells.

As was reported above, dopamine at all effective concentrations resulted in hyperpolarization of the isolated RPeD1 cells (Figs. 10, 11a). The hyperpolarizing effects of 0.1 mM L-DOPA were comparable with the effects of dopamine at 0.1 and 1 μ M concentrations (Fig. 10). The addition of 0.1 mM L-DOPA to 1 μ M, 10 μ M and 100 μ M Ringer’s solutions containing dopamine ($n = 6$) invariably resulted in a sustained depolarization (5 ± 3 mV) and in reoccurrence

of firing lasting for the entire period of observation (5 min and longer, Fig. 11d). Frequency of the discharge slowly declined from 54 ± 9 spikes/min during the first minutes to 46 ± 5 spikes/min after 5 min of L-DOPA application. The second, inhibitory phase of L-DOPA effect was not observed. Changing the superfusate to dopamine alone caused hyperpolarization.

We thus conclude that the first phase of L-DOPA action is a direct excitatory effect of the precursor, whereas inhibition results from L-DOPA conversion to dopamine.

Discussion

This electrophysiological study shows the remarkable responsiveness of a single, isolated aminergic neuron to treatment with the immediate metabolic precursors of dopamine and serotonin. Changes in firing were observed in all individual, identifiable snail neurons examined here. The results of experiments on isolated neurons show that even at the single cell level, a mechanism exists that allows for profound physiological activity of monoamine precursors.

Firing is adjusted to the level of neurotransmitter synthesis

According to neurochemical studies, treatments with either L-DOPA or 5-HTP result in increased content of the respective aminergic neurotransmitter measured in the entire brain, in appropriate single neurons and in vesicles (Colliver et al. 2000; Cottrell and Powell 1971; Fickbohm et al. 2005; Kerkut et al. 1967; Lynn-Bullock et al. 2004; Marinesco et al. 2004a; Pothos et al. 1996, 1998; Poulain et al. 1986).

Using isolated neurons, we have found that the late, inhibitory action of L-DOPA on the dopaminergic neuron as well as the entire excitatory action of 5-HTP in serotonergic neurons are mimicked by their respective neurotransmitters and eliminated by the inhibitor of AAAD NSD-1015. Specifically, serotonin excited isolated PeA neurons and the excitatory effect of 5-HTP in these cells was abolished by NSD-1015. Dopamine inhibited RPeD1 cells, and the inhibitory phase of L-DOPA action was abolished by NSD-1015. Moreover, in serotonergic neurons, long-term treatment with NSD-1015 caused weak but significant decreases in firing. These findings indicate that: (1) the effect of a precursor is completely or partially mediated by the neurotransmitter itself, and (2) the electrical activity of isolated cells is affected by changes in the rate of neurotransmitter synthesis.

This raises the question: where and how does the transmitter exert its influence upon the electrical activity of an isolated neuron? We suggest that newly synthesized neurotransmitter is released from the cell and is thus capable of activating autoreceptors, creating a feedback cycle.

An effective transmitter concentration occurs in the immediate environment of isolated neuron

Our neurotransmitter release hypothesis is supported by our experiments using the inhibitor of the vesicular monoamine transporter reserpine, which prevented the 5-HTP stimulated firing. Although reserpine might cause other effects such as the exocytotic release of serotonin (Mundorf et al. 2000), our hypothesis received further support from experiments using antagonists of the respective monoamine receptors.

In isolated neurons, both dopaminergic and serotonergic, the antagonists effectively blocked the effects of their respective neurotransmitters and abolished the effects of the L-DOPA and 5-HTP application. That is, the antagonist of serotonin receptors, mianserin, blocked the excitatory effect of 5-HTP on PeA neurons, and, correspondingly, the antagonist of the D2 dopamine receptors sulpiride blocked the hyperpolarizing effect of L-DOPA on the dopaminergic RPeD1. Further, we have shown that L-DOPA-induced hyperpolarization can be prevented by pretreatment of the dopaminergic cell with dopamine itself. Serotonin at higher concentrations similarly masked the effect of 5-HTP. These results are consistent with the idea that precursor effects are mediated by the neurotransmitter release, presumably from the cell body, and subsequent activation of membrane autoreceptors.

These findings are also consistent with previous carbon electrode or “sniffer neuron” studies that demonstrated the neurotransmitter release from the neuronal cell body. These studies examined the release of serotonin in leeches (Bruns and Jahn 1995; De-Miguel and Trueta 2005), that of dopamine in snails (Chen et al. 1995; Spencer et al. 2000) and rat (Jaffe et al. 1998), and that of noradrenalin in rats (Chen et al. 2008).

Quite remarkably, in our experiments, the exposure to a serotonin receptor antagonist caused weak but significant hyperpolarization observed even in silent isolated serotonergic neurons. In active and silent dopaminergic neurons, sulpiride had a depolarizing effect. Both effects are again consistent with the known effects of the neurotransmitters serotonin and dopamine on the respective cells. They also agree with the ability of 5-HTP to excite silent serotonergic neuron. Taken together, these observations indicate that tonic spike-independent release of neurotransmitter may occur in silent invertebrate neurons. Quantal spike-independent release of neurotransmitter has been demonstrated and intensely studied previously in the mammalian brain [see (Bouron 2001) for review]. We now show that in the immediate environment of the neuron, an effective transmitter concentration occurs that can vary with the intensity of neurotransmitter synthesis. Thus, a mechanism that adjusts firing to the rate of transmitter synthesis can indeed operate at the single cell level.

L-DOPA may exert direct membrane effects

The immediate, excitatory response of the isolated dopaminergic cell RPeD1 to L-DOPA contrasted with the inhibitory response of the cell to treatment with dopamine. The excitatory effect of L-DOPA was not blocked with the dopamine receptor antagonist and it was enhanced by pretreatment with NSD-1015 or with dopamine. All of these effects suggest that, in the snail brain, L-DOPA is able to directly affect membrane events. This finding is consistent with the reports of L-DOPA directly affecting the rat brain (Misu et al. 1996).

Some evidence indicates that L-DOPA may exist in the extracellular space. Non-enzymatic extracellular synthesis of L-DOPA has been demonstrated in mammals and underlies the poisoning effect of ecstasy (Breier et al. 2006). Further, some catecholamine-producing neurons of the mammalian brain have been described as not being able to synthesize L-DOPA. It is hypothesized that they utilize the precursor produced by and released from neurons of a different subpopulation (Kitahama et al. 1990; Touret et al. 1991; Balan et al. 2000; Ershov et al. 2002; Ugrumov et al. 2004).

Differences in the responses of PeA and RPeD1 neurons to respective neurotransmitter precursors

A major difference between the excitatory effects of L-DOPA and 5-HTP was that the action of the former was decarboxylation-independent, whereas that of the latter was decarboxylation-dependent. No direct membrane action of 5-HTP comparable to that of L-DOPA has been demonstrated. Many more target cells should be examined to decide conclusively if the absence of a polarizing effect of the serotonin precursor on snail neurons is a general effect.

Responses of RPeD1 and PeA neurons to application of their respective neurotransmitters were opposite; RPeD1 responded with inhibition whereas PeA responded with excitation. The difference is hardly fundamental—it might be due to peculiar functions of the cells selected for this study. The RPeD1 cell is a multi-action, multitarget interneuron (Winlow et al. 1984) involved in various functions such as control of neurohormone release (Benjamin 1984) and initiation of the respiratory rhythm (Syed and Winlow 1991). It seems reasonable for this neuron to employ a mechanism that prevents it from being involved in sustained excitation. The late hyperpolarizing action of L-DOPA (actually, that of dopamine) plays this role well. In contrast, neurons of the PeA cluster seem to be involved in modulatory support of a long-lasting behavioral event, locomotion. The cells are known to deliver serotonin to various locomotor organs of *L. stagnalis*, namely, the ciliated epithelium of the sole and locomotor muscles (Syed

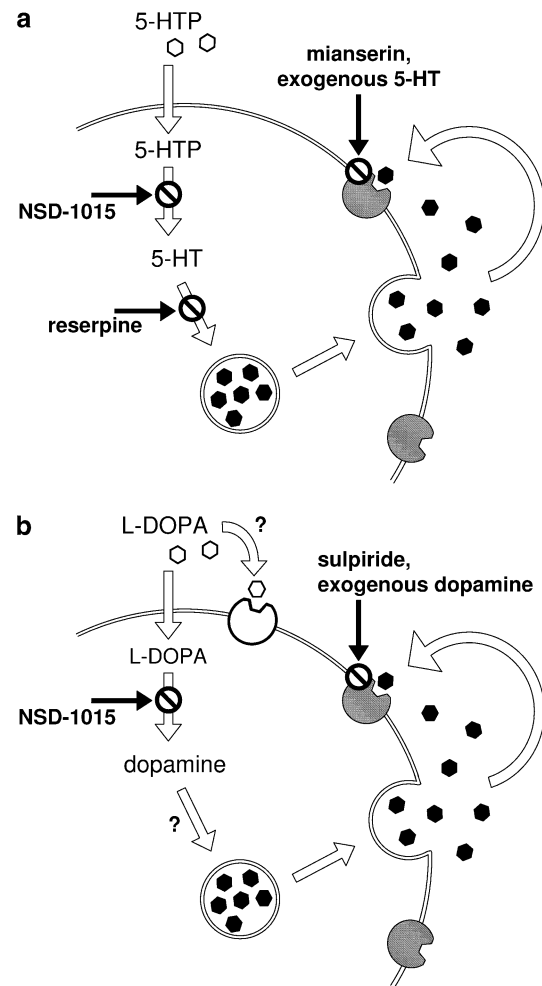


Fig. 12 Schematic representation of the mechanisms underlying precursor effects on the firing of isolated neurons. **a** 5-HTP acts only indirectly through its conversion to serotonin on the firing of isolated serotonergic neuron PeA. The following drugs abolish the excitatory effect of 5-HTP: the inhibitor of AAAD NSD-1015, the inhibitor of the vesicular monoamine transporter reserpine, the serotonin receptor antagonist mianserin and exogenous serotonin at higher concentration. Thus, enhanced synthesis and vesicular release of serotonin with subsequent activation of excitatory autoreceptors underlie 5-HTP effects on firing of isolated serotonergic neuron PeA. **b** L-DOPA acts directly (right) as well as indirectly, through its conversion to dopamine (left) on the firing of the isolated dopaminergic cell RPeD1. Aromatic amino acid decarboxylase (AAAD) inactivation with m-hydroxybenzylhydrazine (NSD-1015), as well as blocking dopamine autoreceptors with sulpiride, results in enhancement of direct (excitatory) effects and abolishment of the inhibitory ones that are mediated by dopamine release. Exogenous dopamine also masks the inhibitory phase of L-DOPA action

and Winlow 1989). Studies on PeA homologs in the swimming marine gastropods *Clione limacina* and *Aplysia fasciata* have shown that strong, long-lasting excitation of these pedal serotonergic neurons is correlated with locomotor arousal (Kabotyanski et al. 1990; Kabotyanski and Sakharov 1990; Satterlie 1995). It seems natural for these neurons to be capable of generating a sustained discharge that can maintain the functions of the locomotor system.

Independent labs have shown that in molluscs, the serotonergic neurons (even those belonging to different networks), are coupled through excitatory chemical and electrical connections. They appear to form a “distributed arousal network” that may underlie the fast behavioral arousal of an animal (Chistopolsky and Sakharov 2003; Dyakonova 2002; Fickbohm and Katz 2000; Marinesco et al. 2004a; Sakharov et al. 1989; Zakharov et al. 1995); for discussion see (Gillette 2006; Sakharov 1990). A positive feedback mechanism that operates at the single cell level fits well to this general idea.

On the other hand, there needs to be another mechanism to protect the cell from explosive behavior with out-of-control firing rates. Indeed, isolated cells do not show an explosive increase in firing rate. This means that a still unknown negative feedback path may also work at the single cell level.

Positive feedback paths within the serotonergic system that are characteristic of molluscs are highly contrasted with vertebrates that have coupling through widespread inhibitory connections between 5-HT neurons within and between their raphe nuclei. Unlike those in molluscs, vertebrate serotonin autoreceptors are inhibitory. One might expect, therefore, that the relationships between synthesis and firing rate in vertebrate serotonergic neurons would be more similar to those described here for RPeD1 cells.

In conclusion, our results demonstrate that both effects, direct (as in the case of L-DOPA) and metabolic (in the cases of 5-HTP and L-DOPA) actions, may be involved in a precursor’s effects on the firing of isolated cells (Fig. 12a, b). 5-HTP acts only indirectly through its conversion to serotonin on the firing of isolated serotonergic PeA neurons (Fig. 12a). The excitatory effect of 5-HTP is abolished by the inactivation of AAAD, the inhibition of the vesicular monoamine transporter, the blockage of serotonin receptors, or the application of exogenous serotonin at higher concentrations. Thus, enhanced synthesis and vesicular release of serotonin with subsequent activation of excitatory autoreceptors underlies the 5-HTP effect on the firing of isolated serotonergic neurons. In contrast to 5-HTP, L-DOPA acts also directly as well as indirectly through its conversion to dopamine on the dopaminergic RPeD1 neuron (Fig. 12b). AAAD inactivation with m-hydroxybenzylhydrazine (NSD-1015), as well as the blockade of dopamine autoreceptors with sulpiride, results in enhancement of direct (excitatory) effects and the abolition of the inhibitory effect. It seems likely that the metabolism of the exogenous precursor substance (either L-DOPA or 5-HTP) and subsequent increases in neurotransmitter synthesis result in enhanced spike-independent and spike-dependent extrasynaptic release of the respective neurotransmitter, which can then affect autoreceptors and firing rates. The question of whether enhanced intravesicular neurotransmit-

ter content can fully explain the increase in spike-independent neurotransmitter release caused by neurotransmitter precursor remains to be elucidated. Numerous intracellular systems are known to modulate spike-independent vesicular exocytosis (Bouron 2001), and involvement of other mechanisms connecting synthesis and release cannot be excluded. Our findings about the role of neurotransmitter synthesis in the regulation of firing rate and extrasynaptic exocytosis may add a novel perspective to current thinking in the field of cellular neurobiology.

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