RELATIONSHIP BETWEEN THE RELEASE AND UPTAKE OF NORADRENALINE BY RAT BRAIN SYNAPTOSOMES IN THE FORMATION OF DEFENSIVE CONDITIONED REFLEX

A. S. BAZYAN and R. I. KRUGLIKOV

Laboratory of Neurochemical Mechanisms of Conditioned Reflex Institute of Higher Nervous Activity and Neurophysiology
Butlerova 5a, 117865 Moscow, USSR

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Abstract. The defensive conditioned reflex with two-way avoidance was developed in rats in a shuttle-box. Immediately and 30 min after learning the animals were decapitated and synaptosomes were isolated from the whole brain and brain cortex. Using [14C] and [3H]-noradrenaline (NA), the processes of uptake, spontaneous and potassium depolarization-induced (60 mM KCl) release of NA by brain synaptosomes of trained and control animals were studied in vitro. Immediately after learning the inhibition of NA uptake was observed, but 30 min following learning the ability of synaptosomes to take up NA was recovered to the initial level. Thirty minutes after learning the potassium depolarization-induced release of NA previously taken up by synaptosomes increased as compared to the control. NA spontaneous release did not change under these conditions. With the increase of Ca\textsuperscript{2+} concentration in the incubation medium from 1.2 mM to 2.4 mM, the induced NA release from brain synaptosomes of trained and control animals regularly increased. Changes in NA release and uptake are specific for the development of conditioned reflex.

INTRODUCTION

As most researchers believe, the formation and fixation of temporary connections is based on the enhancement of synaptic transmission efficiency. An important role in the mechanisms of enhancement of synaptic
transmission efficiency is played by the relationship between the release and inactivation of the mediator. The enhancement of the induced mediator release determines such effects as postsynaptic responses with the increase of nervous impulsation frequency and postsynaptic potentiation (13). The inhibition of mediator inactivation causes the potentiation of postsynaptic responses (15).

Of special importance in the formation and fixation of temporary connections is the catecholaminergic system of the brain. Catecholamines are supposed to be a neurochemical substratum of reward and reinforcement (6, 20). It is believed that catecholaminergic synapses are involved in the connection between conditioned and unconditioned stimuli (11). It may be that this function of catecholaminergic mechanisms is connected with stable changes in the chemoreactive properties of neuron membranes under the action of noradrenaline (NA) and dopamine (16). Therefore it seemed interesting to study the effect of formation of conditioned reflex on the efficiency of adrenergic synaptic transmission in the brain.

The purpose of this work is to determine the effect of the formation of defensive conditioned reflex on the release and uptake of NA by rat brain synaptosomes.

METHODS

Formation of conditioned reflex. The defensive conditioned reflex (CR) with two-way avoidance was developed in a shuttle-box in male rats weighing 180–200 g. Flickering light served as a conditioned stimulus. On the 5th second of its action the box floor was fed with intermittent electric current. The simultaneous action of light and current continued till the animal ran into a dark “safe” chamber. An interval between trials was 30 s. In course of trials the conditioned reflex of avoidance (running away to a “safe” chamber) in response to the action of light was developed till the exposure to the electrodermal stimulus. The reflex was considered formed if five conditioned reactions (avoidances) in six consecutive trials were shown by animals for 15–20 min of training (16–25 combined exposures). The “active” control (AC) included animals exposed to the same number of light and electrodermal stimuli as the experimental ones, but not in combinations. In the last six tests AC animals were exposed to light only. The “passive” control (PC) were animals exposed to nospecial influences.

Subcellular fractionation. The animals were decapitated immediately and 30 min after the exposures. The material obtained from experimental, AC and PC animals was analyzed simultaneously. Synaptosomes from
the whole brain (without cerebellum) or from brain cortex were isolated by Hajès method (8). Brain tissue was homogenized in 18 ml cold buffer (0.3 M sucrose, 0.01 M tris-HCl pH 7.4) and centrifuged (1500 xg, 10 min, in centrifuge K-24, GDR). The supernatant was centrifuged (9000 xg, 20 min), the sediment was resuspended in 5 ml of 0.3 M sucrose, then placed on the layer of 0.8 sucrose (20 ml) and centrifuged (9000 xg, 25 min). Then the suspension of synaptosomes was collected in the upper layer of 0.8 M sucrose (10 ml) and diluted in 0.1 M sucrose to a concentration of 0.3 M. Synaptosomes were obtained in the sediment by centrifugation at 20 000 xg for 30 min and resuspended in cold Krebs-Ringer medium (NaCl — 132 mM, KCl — 4.8 mM, MgCl₂ — 1.3 mM; NaH₂PO₄ — 1.2 mM, glucose — 10 mM, CaCl₂ — 1.2 mM, pargylin — 0.02 mM, tris-HCl — 20 mM, pH 7.4). The protein content was determined by the method of Lowry.

Study of NA uptake. Samples (250 µg protein/ml solution) of protein were stored for 10 min at 0°C, preincubated for 15 min and then incubated for 10 min at 37°C in the presence of 0.25 µCi of DL [¹⁴C] NA (specific radioactivity 35 Ci/mmol, "Amersham" England). The labelled NA was diluted with unlabelled L-NA to final concentrations of 10⁻⁴, 3.5 × 10⁻⁵ and 7 × 10⁻⁶ M. After incubation the samples were centrifuged (20 000 xg, 1 min, centrifuge T-24, GDR). The sediment was washed five times by resuspension and sedimentation in Krebs–Ringer medium at room temperature (25°C). The sediments were dried on air and then solved in 1 ml of 1 M NaOH (45°C, 1 h) and 0.1 ml solution was placed in a 10 ml mixture of scintillating liquid (PPO — 4 g, POPOP — 0.1 g, toluene — 1 l) with ethanol (7:3). Radioactivity was estimated with a scintillation counter (SL-30, "Intertechnique", France). Specific radioactivity was expressed as dpm/1 mg of protein, in 1 ml solution.

Determination of NA release. DL-[³H] NA ("Amersham", England, spec. radioactivity 8.8 Ci/mmol) was used in the experiments. The labelled NA was diluted with unlabelled L-NA to a final concentration of 5 × 10⁻⁸ M. To each sample 0.2 µCi of [³H] Na was added. After incubation synaptosomes with labelled NA were subjected to a series of washings and a sediment of synaptosomes was obtained which was carefully solved in 1 ml of incubation medium (37°C) and 0.1 ml of the solution was immediately, without suspension of sediment, added into the scintillating mixture (control for adsorption). After 2 min incubation of the sediment (37°C) with shaking (no exfoliation of the sediment from the wall was observed) 0.1 ml of the supernatant was collected (for determination of spontaneous release). For depolarization of synaptosomal membranes the incubation medium was supplemented with 0.1 ml 495 mM KCl (final concentration 60 mM), incubated, and two 0.1 ml samples
of supernatant were taken with an interval of 2 min. Since the neurotransmitter release is Ca\(^+\) — independent, the effect of different Ca\(^2+\) concentrations in the incubation medium on NA release from nerve terminals in vitro was studied. Simultaneously, to study in parallel samples the dynamics of NA spontaneous release, three 0.1 ml supernatant samples were taken with an interval of 2 min. The results were calculated for corresponding volumes of the medium subtracting the control for adsorption. After NA release determination of the radioactivity in synaptosomes was measured. NA uptake in these samples was estimated as the sum of radioactively released in the incubation medium and radioactivity remaining in synaptosomes. The spontaneous and induced release was expressed as a percentage of the calculated \(^3\)H NA in the samples containing 1 mg protein in the 1 ml solution, a requirement necessary in secretion studies.

Statistical treatment of the results in each case was carried out according to Student's criterion (T).

RESULTS

As it is evident from Table I, immediately after the formation of conditioned reflex the NA uptake at conc. \((7 \times 10^{-6} \text{ M})\) is inhibited by about 20\% as compared to both control groups. The non-contingent use of stimuli (AC) causes no changes in the uptake as compared to the "passive" control. These changes are not observed at 3.5 \times 10^{-5} and 10^{-4} M NA concentrations. After 30 min the ability of rat brain synaptosomes to take up \(^14\)C NA is recovered to the initial level. The lack of changes between the trained and control rats 30 min following the formation of conditioned reflex was also observed in case of \(^3\)H NA uptake by synaptosomes from brain cortex (see uptake in Table II).

As can be seen in Table I, the radioactivity of the samples falls as NA concentrations are increased. This is due to the fact that the same amount of labelled NA is diluted by different amounts of unlabelled NA. Thus, the percentage of labelled Na is decreased with respect to the total NA content with increasing concentrations, which results in reduction of radioactivity of samples despite a possible increase in the uptake intensity with increasing NA concentrations.

NA release. As it has been shown, an inhibition of NA uptake by rat brain synaptosomes is observed immediately following the formation of conditioned reflex, but 30 min after the formation of conditioned reflex the ability of synaptosomes to take up NA is recovered to the control level. A question arises whether this fact is concomitant with
Table I

Uptake of $^{14}$C NA (dpm 1 ml/mg protein/1min) by rat brain synaptosomes in controls and after formation of conditioned reflex (means ± SEM)

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>Immediately after exposure</th>
<th>30 min after exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10$^{-4}$M NA number of animals</td>
<td>3.5 × 10$^{-5}$M NA number of animals</td>
</tr>
<tr>
<td>PC</td>
<td>7605 ± 304 6</td>
<td>13 911 ± 565 6</td>
</tr>
<tr>
<td>AC</td>
<td>7669 ± 314 6</td>
<td>13 645 ± 596 6</td>
</tr>
<tr>
<td>CR</td>
<td>8189 ± 350 6</td>
<td>14 375 ± 650 6</td>
</tr>
</tbody>
</table>

With respect to PC P < 0.001; to AC P < 0.002.

Table II

The effect of formation of conditioned reflex on spontaneous and K$^+$-induced $^3$H NA release from rat brain cortex synaptosomes (M ± SEM)

<table>
<thead>
<tr>
<th>Ca$^{2+}$ mM</th>
<th>Group of animals</th>
<th>Number of animals</th>
<th>Uptake $^3$H NA dpm 1 ml/mg protein/1 min</th>
<th>$^3$H NA release in % of the uptake</th>
<th>Ratio of release</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>II/I</td>
<td>III/I</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>spontaneous 60 mM KCl 60 mM KCl 4 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 min</td>
<td>2 min</td>
<td>4 min</td>
</tr>
<tr>
<td>1.2</td>
<td>PC</td>
<td>5</td>
<td>18 028 ± 521</td>
<td>8.3 ± 0.7</td>
<td>12.4 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>AC</td>
<td>5</td>
<td>17 843 ± 594</td>
<td>7.8 ± 0.6</td>
<td>13.2 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>CR</td>
<td>5</td>
<td>18 321 ± 574</td>
<td>7.9 ± 0.6</td>
<td>14.9 ± 0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>20 247 ± 683</td>
<td>7.4 ± 0.5</td>
<td>15.4 ± 0.7</td>
</tr>
<tr>
<td>2.4</td>
<td>AC</td>
<td>4</td>
<td>21 097 ± 659</td>
<td>7.8 ± 0.6</td>
<td>16.5 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>CR</td>
<td>4</td>
<td>20 773 ± 639</td>
<td>8.1 ± 0.7</td>
<td>23.2 ± 0.7</td>
</tr>
</tbody>
</table>

Uptake calculated, see Methods. K$^+$ induced compared with spontaneous release: * P < 0.01; ** P < 0.001; 2.4 mM Ca$^{2+}$ compared with 1.2 mM Ca$^{2+}$: P < 0.05; P < 0.01; P < 0.001; CR compared with PC and AC: * with PC and AC. P < 0.05; ** with PC. P < 0.001, AC. P < 0.01; *** with PC. P < 0.001, AC. P < 0.01; **** with PC and AC. P < 0.001.
the recovery of the efficiency of NA-ergic synapse to its initial functional state. To answer this question, it was necessary to study NA release at the time point when the uptake ability was already normalized.

Fig. 1. Potassium depolarization induced release of [3H]NA from rat brain cortex synaptosomes 30 min following the formation of two-way avoidance and non-contingent use of stimuli relatively to "passive" control considered as one (AC/PC; CR/PC). 1, passive control; 2, active control; 3, formation of conditioned reflex. A, CaCl₂ concentration in the incubation medium 1.2 mM; B, CaCl₂ concentration in the incubation medium 2.4 mM.

The results of NA release are summarized in Table II. As it is seen, a spontaneous release of NA from synaptosomal sediment makes up 7–8‰ of NA taken up previously by synaptosomes. Potassium induced depolarization of synaptosomal membranes significantly increases NA release in all three groups of animals. With the increase of Ca²⁺ concentration in the incubation medium from 1.2 mM to 2.4 mM, the NA release intensity in the same groups significantly increases.

The formation of conditioned reflex results in the enhancement of potassium depolarization-induced NA release at both Ca²⁺ concentrations as compared to the AC and PC groups.

The non-contingent use of stimuli brings about insignificant changes as compared to PC. At the same time, the formation of conditioned reflex as well as non-contingent use of stimuli do not influence a spontaneous NA release.

Despite the fact that increasing Ca²⁺ concentration results in increasing NA release induced by depolarization, the correlations between the trained and PC animals remain practically unchanged. Two minutes of depolarization cause the increase of release in the trained animals by 1.3–1.4 times, and 4 min of depolarization — by 1.6 times (Fig. 1).

Thus, immediately after the formation of conditioned reflex, a specific inhibition of NA uptake is observed, the initial level being restored.
30 min after the formation of conditioned reflex. At the same time point, in the state of relative rest, nerve terminals from brain cortex of the trained rats do not differ from the control with respect to the parameters studied. But in the activated state — potassium induced depolarization — a specific increase of NA release from synaptosomes of trained rats brains occurs.

DISCUSSION

It is known that NA uptake by the nervous tissue occurs with high and low affinity at \( K_m 4.0 \times 10^{-7} \) and \( 2.5 \times 10^{-4} \) M, respectively (7, 18). The high-affinity NA uptake is characteristic mainly of noradrenergic nerve terminals, 80% of taken up NA being deposited in synaptic vesicles (5). The low-affinity uptake consists of two components — NA transport through the postsynaptic membrane of the adrenergic synapse and non-specific NA uptake by the transmission mechanisms of other neurotransmitter systems (1). In studying NA uptake by synaptosomes at certain concentrations of the transmitter, non-specific NA uptake by non-adrenergic synaptosomes can be observed. It was shown that at \( 8-10 \times 10^{-5} \) M NA concentration there is a 50% inhibition of high-affinity uptake of serotonin, i.e., a non-specific NA uptake by serotoninergic nerve terminals occurs. This NA concentration is 10-fold higher than the concentration used by us. It was also shown that the level of dopamine uptake by noradrenergic nerve terminals is higher than the level of NA uptake by dopaminergic ones. In view of these data it can be suggested that at the NA concentration used by us \((7 \times 10^{-8} \) M\), the high-affinity NA uptake by catecholaminergic synaptosomes mainly occurred.

The data of our studies show that the formation of two-way avoidance reflex in rats leads to the inhibition of high-affinity NA uptake by catecholaminergic, including the noradrenergic nerve terminals. In this connection, it can be suggested that immediately after the development of two-way avoidance the efficiency of adrenergic synaptic transmission is increased. Thirty minutes following the training no changes in NA uptake and spontaneous release by synaptosomes were observed. However, in case of potassium induced depolarization, the NA release from brain synaptosomes of trained animals is enhanced. \( \text{Ca}^{2+} \) is known as one of the main regulators of neurotransmitter release in situ. In our in vitro experiments the induced NA release evenly increased with increasing the \( \text{Ca}^{2+} \) concentration in the incubation medium from 1.2 mM to 2.4 mM, which suggests common mechanisms of NA release regulation in situ and in vitro.
Thus, the formation of defensive conditioned reflex with two-way avoidance leads not only to the inhibition of NA uptake, but also to the enhancement of induced NA release. The resultant increase in the efficiency of noradrenergic synaptic transmission is specific for the development of conditioned reflex and is not observed in AC. This fact is of special interest, since it implies an important role of the non-contingent use of stimuli. It should also be borne in mind that the stress caused by the electrodermal stimulus results in changes of NA uptake by rat brain cortex synaptosomes only in case of 5 min exposure and does not have such effect in case of 2-, 10- and 30-min exposures (9). It is quite possible that in our experiments the total duration of electrodermal exposure of AC animals was insufficient or too long to cause changes in NA uptake by brain cortex synaptosomes.

The enhancement of induced NA release and the reduction of synaptosome capacity for NA reuptake revealed in this work seem to reflect plastic changes in adrenergic nerve terminals occurring during the formation of conditioned reflex. These changes continue displaying a certain dynamics also after the formation of conditioned reflex. This means that the dynamics of intensity of NA release and reuptake possess different time parameters, which may suggest the relative independence of these two processes in synapases.

The effect of learning on the mechanism of high-affinity reuptake was also reported by other investigators (2). In those experiments an increase of high-affinity choline uptake due to learning was observed. The opposite effect, as compared to our results, is caused by the different role of reuptake processes in the mechanisms of adrenergic and cholinergic synaptic transmission. It was also found that immediately following the formation of conditioned reflex, the tyrosine hydroxylase activity in preparations of rat hypothalamus and hippocampus is increased (3), as well as the NA content in rat brain synaptosomes (12), which may enhance NA release. These facts are in agreement with our results.

In the works on the role of brain adrenergic system in the formation and fixation of temporary connections, this system is shown to be involved mainly in the formation of temporary connections (14, 19). In addition, there is evidence in favour of its relation to the processes of consolidation (4, 11). The data of the present work suggest that the NA-ergic brain system is related to the consolidation of temporary connections, although the mechanisms of its participation in this process are still obscure.

The role of reinforcing adrenergic synapses may consist in “linking” and subsequent stabilization of synaptic inputs activated by conditioned and unconditioned stimuli (11). In other words, adrenergic synapse is an
input which is responsible for changes and stabilization of chemoreactive properties of neurons and for enhancement of the efficiency of synaptic input for a conditioned stimulus (10, 17). The results obtained indicate that in the formation and fixation of temporary connections there occurs a specific enhancement and fixation of the efficiency of the reinforcing or "linking" neuron input itself.

REFERENCES


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