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Striatal Level of Regulation of Learned Forepaw Movements in Rats

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Summary

The role of the striatal adenylyl cyclase (AC) and cholinergic systems in the learning and expression of new forepaw movements (reaching with prolonged pushing on a fixed piston) was studied in male Wistar rats. Motor learning processes, prenatal hypoxia, and cholinergic drugs changed the properties of the AC system in the striatum. After learning, the striatal basal AC activity was decreased compared to untrained control rats. In addition, the AC activity was more decreased in animals with a good ability to learn compared to poor learners (up to 31 % and 51 %, correspondingly; $p<0.01$). Rats subjected to prenatal hypoxia (13-14th days of embryogenesis) had a lower ability to learn the new movements requiring tactile control and the striatal AC activity in these rats was 1.8 times higher ($p<0.001$) than controls. *In vitro* application of the cholinergic agonist carbachol (CARB) 10^{-5} M (corresponding to ~ 0.3 μg), as well as the antagonist scopolomine (SCOP) 10^{-5} M (~ 0.3 μg) decreased AC activity in the synaptosomal fraction of the striatum. *In vivo* injections of CARB (0.3-3 μg/1μl) or SCOP (0.3-3 μg/1μl) into the ventral striatum (nucleus accumbens) modified the newly learned sensorimotor skill. After CARB injections the rats performed slower movements with more prolonged pushing. After SCOP the rats could not retain the learned pushing movement. These *in vivo* and *in vitro* data suggest that the cholinergic mediator system of the striatum is involved in learning sensory-controlled forepaw movements as well as the regulation of new motor skills by modulating the AC signal transduction process in the striatum. The data confirmed that modification of the striatal AC system resulted in the modulation of reaching behavior and better expression of the learned reflex.

Key words

Striatum • Cholinergic systém • Adenylyl cyclase • Learning • Reaching movement • Rat

Introduction

It is well known that the basal ganglia are involved in regulating movements (Brooks 1986) but analysis of the functional role of the basal ganglia is difficult because of the heterogeneity of their morphological and neurochemical organization (Graybiel 1995, Graybiel and Ragsdale 1983, Canales and Graybiel 2000). This is why data on the molecular mechanisms of the regulation of function are rather contradictory and why pharmacological studies remain valuable. Pharmacological investigation is useful for elucidating the role of different mediatory systems and membrane components in the regulation of learning and memory during the performance of both innate and voluntary movements. In addition, diseases such as Parkinson's syndrome, Alzheimer's disease, Huntingdon's chorea, ballism and many others may result from imbalances in the main mediators of the forebrain (Albin 1995, Wichmann and Delong 1998), particularly acetylcholine and dopamine.

The study of new skilled movements and the organization of the nerve centers participating in their

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regulation is important for elucidating the central mechanisms that underlie motor activity as well as the development of pathology. Reaching behavior in which a rat is trained to reach into a narrow tube with a forepaw for food pellets has often been used to investigate the neuronal mechanisms of motor learning (Peterson 1934, Dolbakyan *et al*. 1977, Moroz and Bures, 1983, Whishaw *et al*. 1986, Bures *et al*. 1988, Bracha *et al*. 1990). This model allows the analysis of both the motor and sensory-controlled components of a discrete skilled movement (Zhuravin and Bures 1986, 1989, Bures *et al*. 1988, Zhuravin *et al*. 1996, 1999).

It is well known that the acquisition of new movements is accompanied by structural and functional modifications in the CNS and that learning and memory to a great extent depends on plasticity in the nervous system and the properties of various systems of signal transduction. We have examined the properties of the different biochemical systems of the sensorimotor and the limbic brain in the formation of voluntary movements in rats and found correlations between certain biochemical characteristics of the neuron membrane (such as the content of gangliosides, the activities of adenylyl cyclase, 5'-nucleotidase, and acetylcholinesterase) and the ability of the rat to learn instrumental movements (Zhuravin *et al*. 1995, 1996, 1999). The basal ganglia have a wide spectrum of receptor systems that mediate the actions of various agents on nerve cells through the adenylyl cyclase (AC) system. Changes in the level of AC activity accompany the effects of pharmacological agents, learning, and pathological conditions, such as stress and hypoxia, (Okada *et al*. 1994, DeLapp *et al*. 1996, Olianas *et al*. 1996, Nalivaeva *et al*. 1998, Plesneva *et al*. 1998, Zhuravin *et al*. 2000).

During several years we have been studying the role of the cholinergic and other mediatory systems of the sensorimotor brain in the learning and performance of motor functions in rats as well as on the regulatory properties of the AC system under normal and pathological conditions. In the present paper the most important results are summarized. The investigation focuses on the striatum which participates both in the central regulation of motor activity as well as in learning of new skills.

The reported work had four aims. To study AC activity under 1) normal and 2) pathological conditions before and after the learning of new forepaw movements. 3) To study *in vitro*, the effects of a cholinergic agonist and an antagonist on AC activity in the rat striatal synaptosomal fraction. 4) To analyze the influence of intra-striatal injections of the cholinergic agonist carbachol (CARB) and the antagonist scopolamine (SCOP) on the learning and performance of newly learned forepaw movements[1].

Methods

Animals

Three month old male Wistar rats weighing 180-200 g were used. They were obtained from the colony at the Sechenov Institute. Prior to surgery, the rats were housed 4 per cage in a vivarium at 22 °C, natural lighting and freely available water. The animals were only fed in the experimental box with the reward food pellets (mixture of 3 mg sugar and 27 mg starch) or with standard food if it was necessary after training sessions. The rats were maintained at not less than 85 % of their free feeding weight.

In accordance with the aim of the experiments, the animals were divided into several groups.

Experimental series 1. AC activity after motor learning: Untrained controls (n=10); rats trained to push on a piston (n=18) divided into those with a good ability to learn (n=9) and those with a poor ability to learn (n=9).

Experimental series 2. AC activity and learning ability after prenatal hypoxia: Control rats divided into untrained (n=10) and a trained group (n=20). Rats subjected to prenatal hypoxia divided into an untrained (n=9) and a trained (n=18) group.

Experimental series 3. AC activity in the synaptosomal fraction of the striatum during *in vitro* administration of carbachol or scopolamine. Untreated controls and drug treated groups (n's=9).

Experimental series 4. Behavioral effects of striatal injections of cholinergic drugs: Rats trained only to touch the piston (n=21) and rats trained to push on the piston (n=26). In accordance with the injection procedure, animals were divided into several groups: Uninjected operated controls (n=14); saline injected controls (n=15; number injections - m=28); CARB injected (n=10, m=35) SCOP injected (n=8, m=18).

Training paradigm

The animals were adapted to the experimental conditions during one week. Each day they spent 15 min in the experimental chamber. After two days of food deprivation, during one week, in a daily 15 min session, the rats were trained to reach into a horizontal feeding tube (11 mm in diameter) for food pellets with the

preferred forepaw. The tube was in the wall of the experimental chamber, 60 mm above the floor.

Further training used a similar tube that had a piston 17.5 mm inside. The rats had to push on the piston until a criterion was fulfilled. Some rats were trained only to touch the piston (criterion time (CT) = 0 ms). Other groups of rats were trained to push the piston for CT≥50 ms. When the learned movement was performed correctly, the food reinforcement was given automatically from a hole 15 mm below the tube. The method and the equipment are modified from Zhuravin and Bures 1986, 1989 and are described in detail in Dubrovskaya and Zhuravin (1995). Training sessions were self-paced and consisted of 64 reaches which took 2-5 minutes to complete. The rats were trained for 30-45 sessions before surgery or the biochemical experiments. The two to four experimental sessions were performed at the same time each day. The rats were judged to have had a good ability to learn when their the average pushing time in a session was ≥50ms for 6 or 7 successive sessions ($p<0.05$; t-tests). The other rats were considered to have a poor ability to learn and their average pushing time was not significantly altered across training (Fig. 1, bottom).

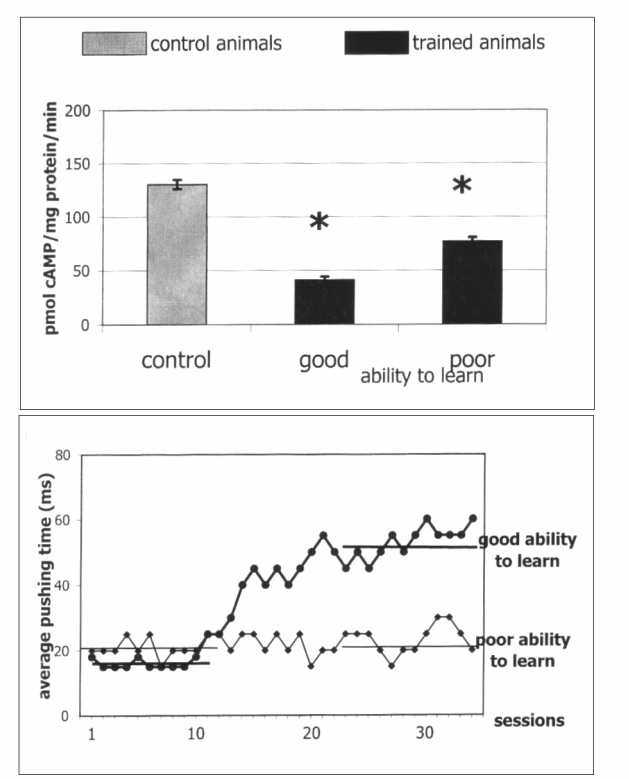

Fig. 1. *Activity of adenylyl cyclase in the striatum after training. Top: Activity of adenylyl cyclase in the striatum of control and trained rats.Ordinate: AC activity, pmoles cAMP/mg of protein/min. Light columns: enzyme activity (mean ± S.E.M.) for the group of control rats. Dark columns: enzyme activity (mean ± S.E.M.) for the group of animals with a good and a poor ability to learn the reaching movement with pushing. * - statistically significant (p<0.001) differences between the experimental and control groups. Bottom: Training of two rats with a good and a poor ability to learn. Abscissa: number of sessions; ordinate: average time of pushing (ms). Horizontal lines – average time for period under the line.*

The experiments were controlled by an IBM PC using a program that measured 1) the number of all reaching movements in a session, 2) the number of the correct (reinforced) reaching movements in a session, 3) the duration of a session, 4) the number of reaching movements per minute ('reaching rate'), 5) the number of correct reaching movements per minute ('correct reaches'), and 6) the average time the forepaw was touching or pushing the piston ('contact time'). The reaching movements were classified as fast (<50 ms) or slow (≥50 ms).

Surgery

Trained animals were anesthetized (Nembutal, 40 mg/kg, i.p.) and mounted in a stereotaxic apparatus (STM-3, Russia) for surgery. Two stainless steel injection guide cannulae (external diameter 0.9 mm) were bilaterally implanted in the ventral striatum (VS) above the nucleus accumbens (AP=–1.5 mm; L, R=1.5 mm; DV=5.5 mm), using the stereotaxic atlas of Fifkova and Marshala (1967). The cannulae were cemented to the skull with protacryl.

Injections of drugs

About a week after surgery, training resumed. When reaching performance returned to pre-surgical levels, drugs were injected through the cannulae. Injections were given outside of the experimental chamber. The animals were injected into the VS with 0.03, 0.3 or 3 μg of CARB or 0.3 or 3 μg SCOP (Sigma, USA) dissolved in 1 μl of saline. In control experiments the animals were injected only with 1 μl of saline.

The injections were given by inserting a needle (external diameter 0.5 mm) into a guide cannula so that the needle protruded a known distance. A ~70 mm length of teflon tubing connected the needle to a Hamilton syringe (Hamilton, Aldrich Chemical Company, USA). Multiple injections were made on different days through the same cannula. Successive injections were 0.5 mm

deeper than the previous (target: DV=6.0-7.5 mm) to insure fresh contact with the interstitial space. The injection procedure usually took about 3 min. The animals were tested in the experimental box before every injection, and 60, 120, 180 min, and for two successive days after every injection.

After all experiments the rats were anesthetized (Nembutal, 80 mg/kg, i.p.) then decapitated. The skull with the brain was stored in 10 % formalin solution then prepared for histological sectioning on a freezing microtome. The positions of the implants and the injection tracks were verified histologically.

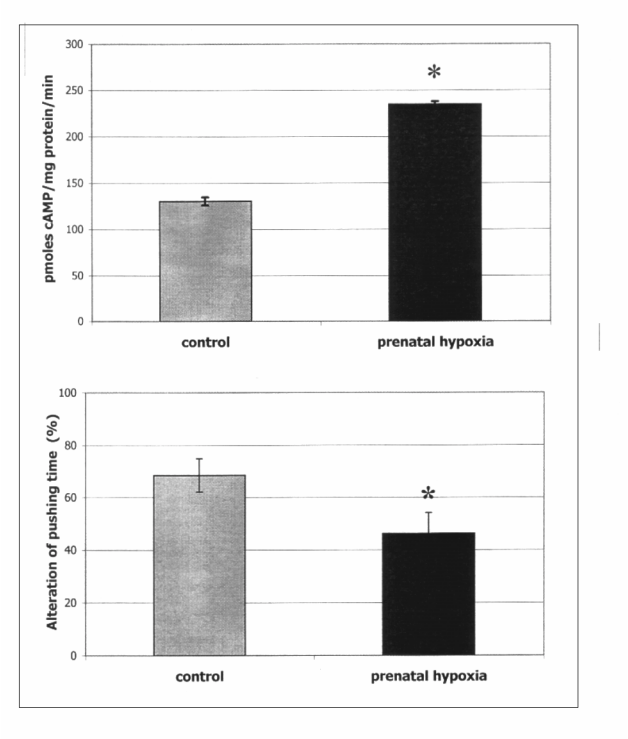

Fig. 2. *Activity of adenylyl cyclase in the striatum and motor training under normal and prenatally hypoxic conditions. Top: Activity of adenylyl cyclase of control rats and rats subjected to prenatal hypoxia. Ordinate: AC activity, pmoles cAMP/mg of protein/min. Light columns: enzyme activity (mean ± S.E.M.) for the group of control rats. Dark columns: enzyme activity (mean ± S.E.M.) for the group of rats subjected to prenatal hypoxia. *- statistically significant (p<0.001) differences between the experimental and control groups. Bottom: Comparison of learning to push the piston in the groups of control rats and rats subjected to prenatal hypoxia. Ordinate - alteration of pushing time (%): light columns - for control rats (mean ± S.E.M.); dark columns – for rats subjected to prenatal hypoxia (mean ± S.E.M.). *- statistically significant (p<0.001).*

Preparation of synaptosomal fractions

Rats were decapitated, after cervical dislocation and the striatum were dissected from the brain. All preparations were performed at 4°C. The dissected tissue was homogenized in 0.04 M tris-HCl (pH 7.4) and centrifuged 10 min at 1 000 g. The supernatant was then centrifuged for 20 min at 20 000 g. To prepare synaptosomes (Hajos 1975), the pellet was resuspended in 0.32 M sucrose in 0.04 M Tris-HCl (pH 7.4) and centrifuged for 1 hour in a sucrose gradient of 0.32, 0.8 and 1.2 M sucrose in 0.04 M Tris-HCl (pH 7.4) at 100,000 g. Synaptosomes were then washed twice with 0.04 M Tris-HCl (pH 7.4) in the same conditions (100 000 g for 1 h). The sucrose-free synaptosome pellet was suspended in 0.04 M Tris-HCl (pH 7.4) and analysed for AC activity.

Adenylyl cyclase assay

The activity of AC was assayed in the presence of IBMX (an inhibitor of phosphodiesterases) according to the method of Salomon *et al*. (1974). The incubation medium (volume 50 ml) contained (mM): 50 Tris-HCl (pH 7.4 at 37 ^{o}C), 5 $MgCl_2$, 1 cAMP, 20 creatine phosphate, 0.1 ATP, 1 mg/ml creatinephosphokinase and [α-^{32}P]ATP (0.5-1.0 μCi per sample). The samples were incubated at 37 °C for 10 min. The effect of CARB and SCOP (10^{-6}-10^{-5} M) on AC activity was analyzed by adding these compounds directly to the incubation media.

Prenatal hypoxia

On the 13-14th day of gestation pregnant female Wistar rats were subjected to normobaric hypoxic hypoxia (content O_2=7 %, 3 hours) in a special chamber containing systems for gas control, ventilation and withdrawal of expired CO_2. Their offspring were taken for behavioral experiments when they were adult (3 months).

Data analysis

Statistical evaluation of the data was made using t-tests and ANOVAs.

Results

Training.

At the beginning of training each rat was observed to have an unstable, short pushing time. Contact time (CT) was about 20-30 ms. After several cycles of training, using increasing push-criteria, the pushing time became longer - the average duration of pushing was

more than 50-100 ms. Some pushing times were greater than 250 ms.

Experimental series 1

AC activity after motor learning. The basal level of AC activity was 131.4±4.4 pmoles cAMP/mg protein/min in the synaptosomal fractions from the untrained control rats. After three weeks of learning to push the piston, the striatal basal AC activity was decreased compared to the untrained controls (Fig. 1, top). Furthermore, the AC activity was more decreased in the group of rats that had a good ability learn compared to those with poor learning (up to 31 % and 51 %, correspondingly; $p < 0.01$).

Experimental series 2

AC activity and learning ability after prenatal hypoxia. The adult male rats that were subjected to prenatal hypoxia on the 13-14th day of embryogenesis had 1.8 times higher striatal AC activity than the control group ($p<0.001$; Fig 2 top). In addition, the rats in the hypoxia group had a lower ability to learn the new instrumental movement compared to normal controls ($p<0.001$; Fig. 2 bottom).

Experimental series 3

AC activity in the synaptosomal fraction of the striatum during *in vitro* administration of carbachol or scopolamine: *In vitro* CARB and SCOP (10^{-5} M) inhibited AC activity by 23 % and 28 %, respectively (Fig. 3). The 10^{-6} M concentration of both drugs did not cause statistically significant changes in AC activity from the basal control level.

Experimental series 4

Behavioral effects of striatal injections of cholinergic drugs.

Control animals. The contact time and the reaching rate were unchanged after surgery and after intra-striatal saline injections. The percent of reinforced reaches also did not change in the control groups (Table 1).

Table 1. The percent of change of the average contact time (CT) after injections into the ventral striatum (100 % is the control value, before manipulation). # - $p<0.01$ and * - $p<0.001$.

Contact time (ms)	Without injection (after surgery)		Saline vehicle		Carbachol 0.3-3μg		Scopolamine 0.3-3μg	
	before	after	before	after	before	after	before	after
CT=0	100	101.6±3.1	100	91.3±13.9	100	99.4±10.2	100	100.6±6.6
CT> 50	100	98.6±4.0	100	95.7±7.3	100	113.4±4.6#	100	67.1±5.7*

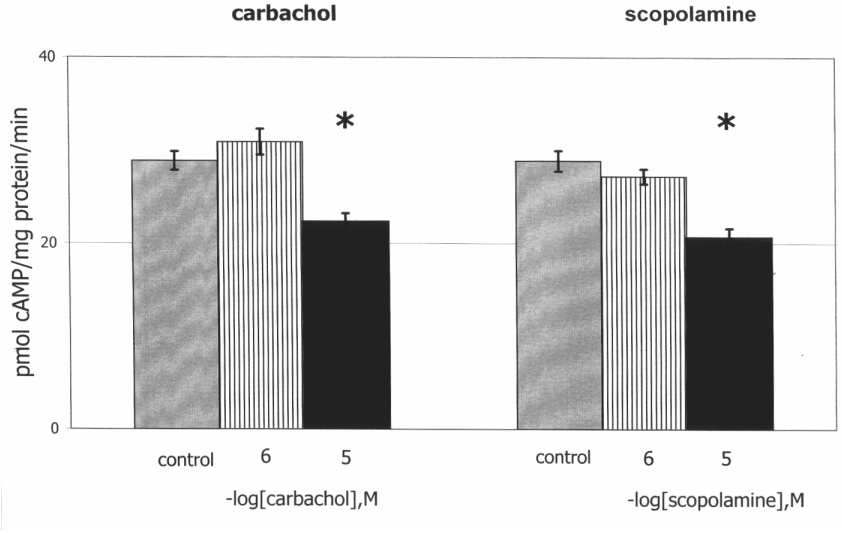

Fig. 3. *The In vitro effect of cholinergic drugs on adenylyl cyclase activity in the synaptosomal fraction from the striatum. Ordinate: AC activity, pmoles cAMP/mg of protein/min. Light columns: control values (mean ± S.E.M.) of AC activity without carbachol or scopolamine. Hatched columns: value (mean ± S.E.M.) of AC activity in the presence of carbachol (left, 10^{-6}, 10^{-5} M) or (black columns) scopolamine (right, 10^{-6}, 10^{-5} M). *- significant ($p<0.001$) differences from the control group.*

Drug injection. The rats that were trained to only touch the piston were unaffected by any doses of the CARB or SCOP injections. There were no statistically significant changes of the reaching rate, the number of slow and fast movements (Fig. 4, top row) or the average piston contact time (Table 1, CT=0). The percentage of slow reaching movements (>50 ms) was similar before (27-35 %) and after (23-33 %) the injections (Fig. 4, top row; see the numbers above the bars).

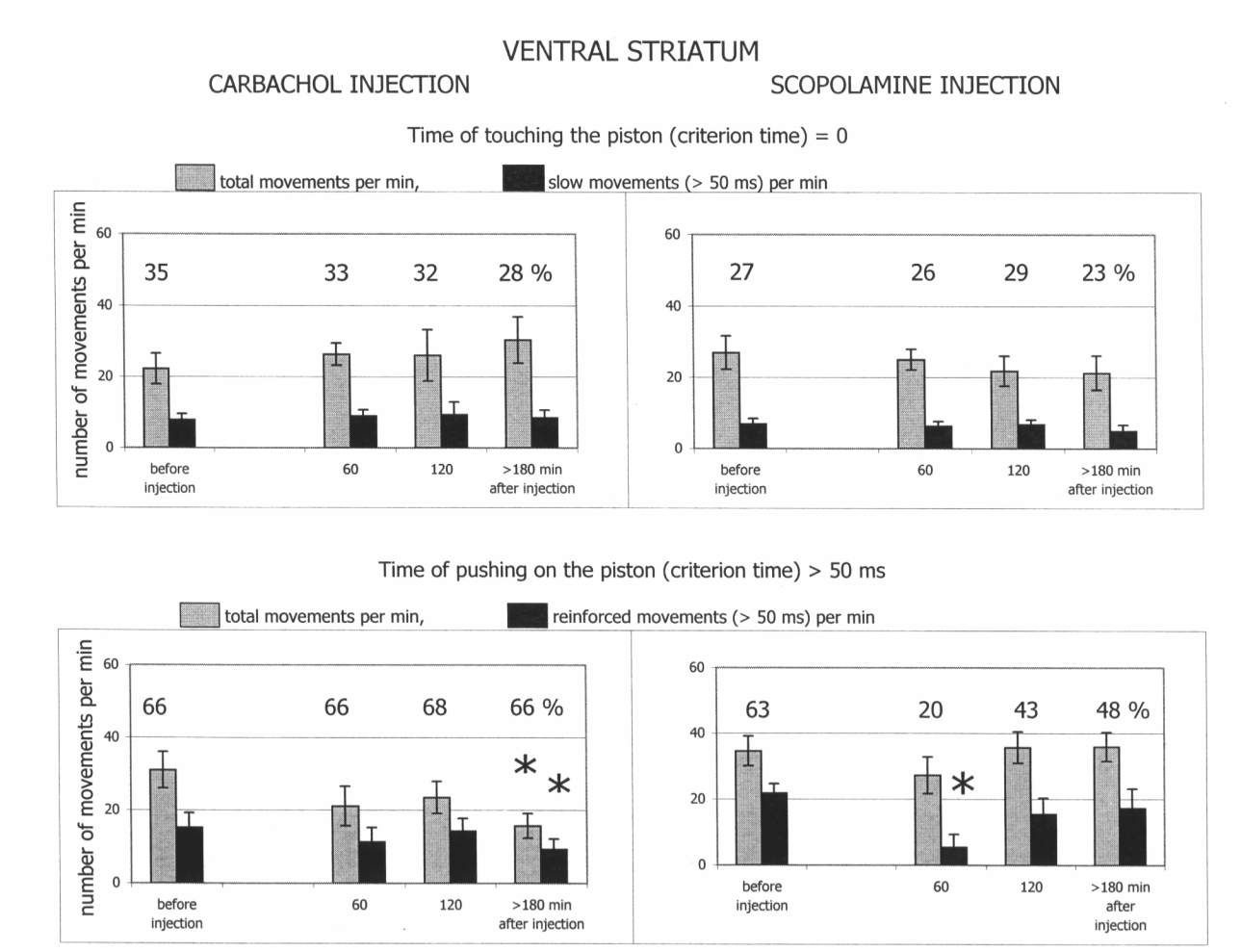

Fig. 4. *The influence of carbachol and scopolamine injections into the ventral striatum on reaching with pushing under different reinforcement criteria. Top row – criterion time of touching the piston 0; bottom row – criterion time of pushing on the piston > 50 ms. Abscissa: time after injection in min. Ordinate: number of movements per min. Light columns: total number of reaches per min in one session (mean ± S.E.M.). Dark columns: number of slow movements (>50ms) per min in the same session (mean ± S.E.M.). The % values above the columns are the average percentage of slow movements in the same session (total number of movements per min is 100 %). *- statistically significant (p<0.01) differences from the level before injections.*

In the group of rats that was trained to push for 50 ms or more, the CARB injections of 0.03 μg also had no effect, but injections of the higher doses of both CARB and SCOP produced quite different results (Fig. 4, bottom row). The reaching rate was decreased after the 0.3 and 3 μg CARB injections. The decrease was maximal 180 min after the injection. Despite the decrease, the percent of correct reaches (68 %) was increased relative to before the injections (61 %) but the difference was not statistically significant. The percent of the average correct (slow) contact time also increased (113.4±4.6 %) and this was statistically significant ($p<0.01$, Table 1).

Although the animals injected with SCOP did not change their reaching rate, by 60 min after the injection they had lost the ability to reach correctly with slow movements (20% correct, Fig. 4, bottom right). The percentage of the average correct (slow) contact time was

also decreased (67.1±5.7 %, $p<0.001$, Table 1). Recovery of the ability to reach correctly began by 120 min after the injection and was complete by the next day.

Discussion

Learned reaching behavior of rats was used to study the cholinergic regulation of sensory-controlled forepaw movements by the striatum. Injections of cholinergic agonists and antagonists disrupted reaching behavior and the severity of the disruption was shown to depend on the character of the pharmacological changes. After training to make prolonged pushes on a piston, the animals made few fast reaches; their pushes were slower, requiring greater regulation of the movements to maintain forelimb muscle tone. This model allowed us to compare the involvement of neurotransmitter and signal transduction systems of the striatum for performing fast and slow, more tactile controlled movements.

Learning to reach with pushing was also shown to be accompanied by significant changes in the biochemical parameters of brain structures such as the content of gangliosides, and the activities of 5'-nucleotidase and acetylcholinesterase (Zhuravin *et al*. 1995, 1996, Plesneva *et al*. 1998, Zhuravin *et al*. 1999). We found AC activity was decreased in the striatum after learning to perform movements with increased sensory control. Moreover, AC activity was lower in rats that were better at learning the movement. These data lead us to suggest that lower AC activity provides a more sensitive regulation of the molecular processes involving the cAMP-dependent signal transduction system in the striatum. The decreased level of AC activity in rats with a good-ability to perform the instrumental reflex might be connected with the better ability of the neuronal network to achieve the regulatory functions of the cholinergic, cAMP-dependent transduction systems. This view was supported by the experiment that examined reaching after experimentally induced pathology. The rats subjected to prenatal hypoxia had a lower ability to learn, and at the same time, they had higher AC activity in the striatum.

It is possible that the striatal AC system might be involved in the process connected with the learning related changes in the striatal cholinergic system. The data on the reaction of the AC system to CARB and SCOP may indicate that the effects of these agents on AC involve inhibitory G proteins. We have shown that the striatal AC system is regulated by such agents as Gpp[NH]p, forskolin, and NaF (Plesneva *et al*. 1998, Zhuravin *et al*. 1999). This regulation is mainly due to changes in properties of the catalytic (enzyme AC) and regulatory component (G-proteins) of the AC system (Sternweis *et al* 1981).

It is a well known hypothesis that acetylcholine and dopamine have opposing functions in the extrapyramidal system (McGeer *et al*. 1978) and that under normal conditions, cholinergic and dopaminergic tone are in approximate balance (Barbeau 1962). In Parkinson's disease, cholinergic tone predominates, while in Huntington's disease, dopaminergic tone predominates (Aquilonius and Sjostrom 1971). The largest subcortical formations of the forebrain - the basal ganglia have prominent enzyme concentrations for the synthesis and degradation of dopamine and acetylcholine (Schell-Kruger 1985). Unlike many other structures of the forebrain, the striatum (the center of sensorimotor integration of the basal ganglia) has its own source of choline - large cholinergic interneurons (Wainer and Mesulam 1990). These interneurons may have excitatory contacts with efferent cells. These efferent cells inhibit subcortical motor structures as well as thalamic nuclei via the globus palidus and the pars reticulata of the substantia nigra. Dopaminergic nigral neurons activate efferent striatal cells that produce GABA and cause inhibition within the globus palidus and the pars reticulata of the substantia nigra. Thus, nigral dopaminergic and striatal cholinergic cells modulate thalamo-cortical relationships in addition to influencing voluntary movements. These and other data have led to a theory that there are two balanced (excitatory and inhibitory) pathways in the regulation of voluntary movements (Groves, 1983, Shapovalova, 1993).

The present results provide evidence that the cholinergic system of the VS is involved in the process of learning sensory-controlled movements and it has almost no role in performing fast, innate movements. Behavioral disturbances were observed for 60 min after SCOP administration into the VS in rats that had learned to make prolonged pushes, whereas the injections caused no changes in fast, touching movements. These data support the hypothesis (Shapovalova *et al*. 1996) that the striatal cholinergic system has an important role in controlled muscle tone. Thus both the dopaminergic (Ellenbroek *et al*. 1988) and cholinergic systems of the nucleus accumbens are involved in controlling forelimb muscle tone.

Taking into account the literature and our data it is necessary to note that the mechanism of CARB and SCOP action on AC is very complex. Our *in vitro* experiments revealed an inhibitory effect of both CARB

and SCOP on AC activity in the striatum of rats. Cholinergic agonists, and in particular CARB, were also reported to inhibit the activity of AC in the striatum (Olianas *et al.* 1983, Login 1997). It was shown that CARB added to caudate slices or synaptosomes stimulates dopamine release, whereas cholinergic antagonists block the dopamine release (Bradford 1986). Thus the inhibitory effect of the drugs on AC can be realized directly through cholinergic receptors or indirectly via dopaminergic receptors (Battaglia *et al.* 1985).

The status of the AC system of an organism is determined by extracellular regulatory signals interacting with different types of receptors, that are coupled to this enzyme via different types of G-proteins. The existence of multiple forms of AC, which possess different regulatory properties in various types of cells and which may also occur in different ratios within these cells might provide for the ability of nervous cells to recognize, transmit and modulate complex external signals contingent on the physiological status of the brain and behavioral loads (Iyengar 1993).

In general, the present data permits us to conclude that the process of motor learning induces the modulation of mediator and signal transduction systems of nerve cells that result in changes of the functional properties of the CNS. In particular, our data confirmed that modifying the properties of the AC system in the striatum resulted in the modulation of behavior and an improved learned reflex.

Appendix

First of all I would like to congratulate Jan Bures on his anniversary and to wish him prosperity and health. Many years ago, the year was 1973, I met Dr. Bures at a meeting in Rostov-na-Donu (Russia) and we had a short conversation. I think it must have been a difficult time for him but nobody could sense this from him. With great pleasure I remember the summer science workshop for young researchers (in the same 1973 year, in the Institute of Physiology, Prague), in which Drs. Bures, Ivan Krekule, and Gustav Brozek presented their talks and experimental software using modern equipment and training programs. Dr. Bures though young (which he manages eternally to appear) was already rather a prominent scientist, famous as a designer of experimental methods. His books (with M. Petran and J. Zahar; and later with O. Buresova and J.P. Huston; with I. Krekule and G. Brozek; and with O. Buresova and J. Krivanek) were the reference books for our University and research groups. My next visit to the Institute of Physiology (Prague) only occurred 11 years later. Naturally, many people wanted to have the top quality training that was offered in the Bures laboratory. In 1984 I was able to work together with Dr. Bures and under his supervision. While there were many specialists in his laboratory, Jan liked to work with his own hands and amazingly, he still had time and kind-hearted attention for everyone. Jan and Olga have the remarkable ability to organize useful and interesting short projects that are parts of their common research interest. From 1984-1995 I visited the Bures lab. Unfortunately the visits were short, lasting from only three weeks to three months, but nonetheless, we managed to complete several published studies. Of course, my research was carried out with the help of the colleagues who have enjoyed working with Dr. J. Bures for a long time (G. Brozek, V. Bracha, I. Krekule, L. Jerabkova, A. Zahalka, Y. Kaminsky, V. Valouskova and many others). There was a friendly, animated spirit and we had meetings and made a trips to thenew and entertaining sites of Prague and the Czech Republic. Since this time I love Prague and the people who are living there. Hope to see you again.

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