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Fos EXPRESSION AND TASK-RELATED NEURONAL ACTIVITY IN RAT CEREBRAL CORTEX AFTER INSTRUMENTAL LEARNING

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Abstract—Learning has been shown to induce changes in neuronal gene expression and to produce development of task-specific neuronal activity. The connection between these two features of neuronal plasticity remains of a great interest. To address this issue we compared distribution of c-Fos expressing and task-related neurons in the rat cerebral cortex following instrumental learning of appetitive lever-press task. The number of Fos-positive neurons was determined immunohistochemically in the retrosplenial and the motor cortex of naive ("control" group), newly trained ("acquisition" group) and overtrained ("performance" group) animals. A significant activation of c-Fos expression was observed in the neurons of the retrosplenial but not motor cortex in the "acquisition" group rats, as compared with the "control" and "performance" groups. In accordance with this c-Fos expression difference, the retrosplenial cortex of the trained animals contained significantly more neurons with lever-press-related activity than the motor cortex. Therefore, the two examined cortical areas showed a parallel between experience-dependent induction of c-Fos and development of task-related neuronal activity. These data support a notion that learning-induced activation of c-Fos is associated with long-term neurophysiological changes produced by training.

Key words: Fos, gene expression, neuronal activity, retrosplenial cortex, motor cortex, instrumental learning.

Learning is known to be accompanied by activation of neuronal transcriptional cascade involved in long-term memory consolidation (Abel et al., 1997; Abel and Lattal, 2001; Kandel, 2001). This nuclear cascade starts with phosphorylation of the transcription factor CREB, which activates a number of immediate early genes some of which (like *c-fos*, *zif/268*) encode inducible transcriptional factors (ITFs) (Guzowski and McGaugh, 1997; McGaugh, 2000). Increased expression of ITFs has been demonstrated in a variety of learning paradigms in various species (for reviews, see Kaczmarek and Chaudhuri, 1997; Herdegen and Leah, 1998; Tischmeyer and Grimm, 1999; Clayton, 2000). Blockade of these genes by knock-down antisense techniques prevents consolidation of the long-term memory (Mileusnic et al., 1996; Swank et al., 1996; Grimm et al., 1997; Morrow et al., 1999; Tolliver et al., 2000). Moreover, mice with a nervous system-specific *c-fos* knock-out show impaired long-term memory formation (Fleischmann et al., 2003). These data suggest the causal relationships between c-Fos-regulated transcription and consolidation of new experience. Based on this evidence, c-Fos has been proposed as a cellular marker of learning-related neuronal plasticity (Anokhin and Rose, 1991; Rylski and Kaczmarek, 2004). However, it is still unclear, what are the implications of such learning-induced ITFs expression, and c-Fos expression specifically, for the functional long-term changes of neuronal activity (Clayton, 2000; Mello, 2004).

One of the long-term neurophysiological consequences of learning is development of neuronal firing patterns that are specifically related to performance of learned behavior (for example, Alexandrov et al., 2001; Gandolfo et al., 2000; Shima and Tanji, 2000; Chang et al., 2002). This phenomenon of experience-dependent "behavioral specialization" of neurons (Shvyrkov, 1986) is well established (Sakai et al., 1994; Goldman-Rakic, 1995; Bear, 1996; Wiener, 1996; Tanaka, 1997; Eichenbaum, 1999; Helekar, 1999; O'Keefe, 1999), but little is known about its molecular bases. However, such behavioral specializations persist over a long time (Margoliash, 1986; Thompson and Best, 1990; Gorkin and Shevchenko, 1991; Chang et al., 1994; Jog et al., 1999), which suggests involvement of changes in gene expression and protein synthesis in the maintenance of this phenomenon (Agnihotri et al., 2004).

One way to test the relationship between learning-induced c-Fos expression and formation of task specific neuronal firing is to compare spatial distribution of these two phenomena in a particular learning task. If the formation of task-related neuronal activity is mediated by activation of c-Fos, then the areas with the highest density of task-related neurons should coincide with the regions of highest c-Fos expression in a corresponding task. The aim of the present work was to test this prediction. Earlier we have demonstrated that learning of appetitive instrumental task results in nearly 10-fold more task-related neurons in the retrosplenial cortex, than in the anterolateral area of the motor cortex (Alexandrov et al., 1991, 2001). In the current work we compared the distribution of behaviorally

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Abbreviations: ITFs, inducible transcriptional factors; NMDA, N-methyl-D-aspartate; NSE-neurons, neurons that demonstrated activity related to non-specific elements of the operant task; PBS, phosphate-buffered saline; SEM, standard error of the means; SE-neurons, neurons that demonstrated activity related to the specific elements of the operant task; UN-neurons, behaviorally unidentified neurons.

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specialized neurons and the distribution of Fos-positive neurons in these two cortical areas of rats after appetitive lever-press learning.

EXPERIMENTAL PROCEDURES

Immunohistochemistry experiments

Animals. Twenty male Long-Evans hooded rats (5–9 months old) were housed in individual cages. They were food-deprived to 85% of their free-feeding body weight and maintained at this level throughout the experiment. Water was available *ad libitum*. All surgical and experimental protocols were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.The number of animals used and their suffering were minimized.

Behavioral training and analysis. All behavioral training took place in an operant chamber of 40×40×50 cm. The chamber was fitted with two automated plastic food-cups in the corners and two wall-mounted levers located in the opposite corners. Food-cups and levers were equipped with photodiodes. A button controlled by an experimenter was located outside of the cage and allowed filling the food-cup at any required time. Lever-presses and food-cup checks by an animal were registered by Ikegami data-recorder DTR 1204x (Nihon Kohden, Kogyo Co., Ltd., Tokyo, Japan).

Training was conducted daily in 30-min sessions. Animals were progressively shaped across days to acquire the lever-press behavior (Kelly and Deadwyler, 2003). The experimenter delivered a food reward to the subject for approaching the food-cup (two days), then for turning away from the food-cup toward the lever (two days), then for moving half a way toward the lever (two days), then for approaching the lever on the distance of less than 1 cm (two days), and ultimately for pressing the lever. All animals acquired independent lever-pressing behavior on the first lever-pressing session. The "acquisition" group animals (n=8) were killed for immunohistochemistry after their first lever-pressing session. The "performance" group animals (n=7) were killed after their fifth level-pressing session. In order to equalize the total number of sessions in the experimental chamber between these two groups the first stage of training (approaching the food-cup) was prolonged up to five days in the "acquisition" group. Animals of the control group (n=6) were kept in their home cages with free access to food and water and killed at the same time as the trained animals.

Behavioral measures included the number of presses completed and the number of food-cups checked, along with the timestamp of each event. To assess instrumental performance, percentage of correct trials (lever-press followed by food-cup check) was calculated:

% of correct trials=Number of lever-presses/Total number of food-cup checks×100.

Mann-Whitney rank sum test was used for analysis of behavioral variables between the "acquisition" and the "performance" groups. Performance improvement in the "acquisition" group animals during the first lever-pressing session was analyzed by Wilcoxon test.

Immunohistochemistry. Seventy-five minutes after the experimental session, animals were overdosed with halothane. Their brains were removed and frozen for analysis. Coronal 20 μm cryostat brain sections were taken through the retrosplenial cortex (−4.0 to −5.0 mm to bregma) and the anterolateral part of the motor cortex (+2.5 to +3.5 mm to bregma) (Paxinos and Watson, 1997). Sets of neighboring sections were processed for c-Fos immunoreactivity and for Nissl-staining. The sections prepared for immunohistochemistry were dried overnight and fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4, for 15 min. Fixed sections were washed (3×5 min) in 0.1 M PBS and placed into a blocking solution (2.5% normal serum/0.1 M PBS) for 30 min. The sections were then incubated in Fos rabbit polyclonal antibody ("Calbiochem," Ab-5, Cat. #PC38, USA), diluted 1:2000 with 0.1 M PBS, for 18 h. The sections were washed (6×5 min) with 0.3% Triton X-100 in 0.1 M PBS, and incubated with biotinylated goat anti-rabbit secondary antibody ("Vector Laboratories," USA) diluted 1:300 in PBS for 2 h. They were then washed (5×5 min) and processed with the 1% streptavidin–biotin complex (PK-6101, "Vector Laboratories") for 1 h. After 4×5 min washes the sections were placed in a solution of 0.06% diaminobenzidine (DAB, Sigma, USA) and 0.003% H_2O_2 for 6 min. The sections were then washed in tap water, counterstained, dehydrated and coverslipped with the mounting medium.

Data analysis. Images of the retrosplenial and motor cortices were digitized at 20× magnification under Olympus BX-50 microscope (Japan) by WV-CP230 camera (Panasonic, Japan) and analyzed using AnalySis 3.0 image analysis software (SiS, Germany). The number of Fos-positive cells was counted in standard frame sample areas (1.0×1.5 mm for the retrosplenial cortex and 1.0×2.0 mm for the motor cortex). Counts were taken from 10 consecutive sections in each rat. The percentage of Fos-positive neurons out of the total number of cells in standard sample areas of the two cortices was calculated based on the number of Nissl-stained cells. The counting was performed by an investigator blind to the experimental group assignment of the animals.

Kruskal-Wallis ANOVA median test and Mann-Whitney rank sum test for pairwise comparisons were used to compare the number of Fos-positive neurons between the groups. Friedman ANOVA and Wilcoxon test for pairwise comparisons were used to identify significant differences in c-Fos immunoreactivity between the brain hemispheres, between the studied cortical areas, and between the cortical layers. A probability level of <0.05 was accepted as statistically significant.

Single unit activity recording experiments

Animals. Ten male Long-Evans hooded rats (5–9 months old), were used in this part of the experiment. Animal housing and treatment were similar to the ones described for immunohistochemical experiments.

Behavioral training. Rats were trained for lever-pressing behavior for five days in the same manner and in the same operant chamber as described for immunohistochemical experiments. Two pairs of levers and food-cups in the operant chamber were used for additional testing of task-specificity of neuronal activity. Such design allowed testing of neuronal activity in respect to similar behavioral goals (e.g. two levers) approached in different places, or similar movements (e.g. turn to the right) that were used to approach different goals (the lever and the food-cup).).

Surgery. Rats were anesthetized with Ketanest (100 mg/kg, i.p.). A midline incision of the scalp was made, the skin and the muscles were retracted, and three miniature screws were fixed in the skull at appropriate locations to anchor the headstage. Holes over the retrosplenial and the motor cortex were drilled at the following stereotaxic coordinates: 4.5 mm posterior to bregma, 1.0 mm lateral to the midline suture and 3.0 mm anterior to bregma, 3.5 mm lateral to the midline suture (Paxinos and Watson, 1997). These coordinates corresponded to the location of cortical areas for c-Fos immunoreactivity measurements (see above). A low-impedance reference microwire was implanted 1 mm anterior to the retrosplenial cortex registration site. Sterile petroleum jelly was applied to the exposed brain surfaces. The screws and the headstage were embedded in the dental cement. Aseptic procedures were applied through the surgery and periodically thereafter.

Single unit recording. After surgery, rats were allowed to recover for 5–7 days with *ad libitum* access to food and water. Extracellular single-unit activity was recorded with glass microelectrodes filled with 2.5 M KCl (impedance of 2–6 MΩ at 1 kHz) and attached to a removable custom-made microdrive assembly (Korshunov, 1995) placed on the headstage.

At the beginning of the experiment, the recording electrode was placed above the cortical surface and then advanced progressively through the cortex until a spontaneously firing neuron was encountered. To be recorded, cells had to have at least a 3:1 signal to noise ratio. Every unit was recorded during 10–20 lever-pressing trials on each side of the operant chamber. An important aspect of the experimental procedure used in this study is that every isolated neuron was tested extensively with additional sets of tests to ascertain whether the cell was selective for behavioral elements learned in the operant chamber. Such sensorimotor tests included placing a rat in different locations of the operant box, touching different parts of a rat's body (including head, paws and whiskers) by the experimenter's hand, allowing the rat to collect food placed on the floor of the operant box. Extracellular action potentials and behavioral tags were digitized at 30 kHz (DL-120 A/D converter, LCard, Russia). Simultaneously, animals' behavior was videotaped with the sound of neuronal firing recorded to the audio-channel.

After the completion of the experiment, negative current (600 μA for 5 s) was passed through the tungsten electrode to visualize registration sites. Animals were then overdosed with halothane, and the brains were taken for histological analysis. Every second 20 μm section from the area of recording was Nissl-stained for verification of the electrode position.

Data analysis. The relation of neuronal firing to elements of operant lever-pressing behavior was analyzed using a custom-developed off-line analysis software (Y. Raigorodsky, A. Krylov). Neuronal firing patterns were evaluated by generating peri-event histograms of firing rates around (±1.5 s) the onset of each behavioral task-relevant event. Neurons were classified in relation to the operant behavior if there was a 50% firing rate change (from the mean firing rate over the entire recording period) during an element of the operant task. Neurons were classified as related to a *specific element* of the operant behavioral task (approach to the lever, lever-pressing, approach to the food-cup, taking food from the food-cup) if their firing rate changes during such behavioral elements were observed in all cases (100%) (SE-neurons). Cells with place-specific firing were also attributed to this class of neurons. Neurons were classified as related to *non-specific elements* of the behavioral task (e.g. turning left or right, lowering or raising the head) if their firing rate changes during such behavioral elements were observed in all cases (100%) (NSE-neurons). Neurons with activity that could not be related to behavioral elements were classified as functionally *unidentified* neurons (UN-neurons). This analysis was conducted in accordance with procedures established in previous studies of single-unit activity of in the cingulate cortex and the anterolateral part of the motor cortex of rabbits and rats (Alexandrov et al., 1991, 2001; Gavrilov et al., 1998; Gorkin and Shevchenko, 1996). Differences in proportions of task-specific neurons between the retrosplenial and the motor cortex were analyzed using χ^2-test.

The mean number of spontaneously active neurons per track was calculated using the cells-per-track technique (Dai and Tepper, 1998; Xu and Shen, 2001), i.e. the recording electrode was passed systematically through a stereotaxically defined block in the retrosplenial cortex and the motor cortex. The theoretical mean number of neurons within the recording boundaries of one track was calculated by the method of representative cylinders (Henze et al., 2000) using Nissl-stained cell density for the retrosplenial and the motor cortices. The radius of a representative cylinder (50 μm) corresponded to the calculated mean distance of optimal extracellular signal recording with microelectrodes in the cerebral cortex (Mountcastle et al., 1957; Favorov and Whitsel, 1988). The height of the representative cylinder corresponded to the cortical thickness. Though, a certain imprecision is typical for this method (Towe and Harding, 1970), it was, however, satisfactory for our purpose of comparing the results of the neuronal recording experiments with the c-Fos expression data.

RESULTS

Behavioral performance

During the final training session, rats of the "acquisition" group learned the lever-pressing behavior. Lever-pressing behavior naturally followed a short period of non-reinforced lever-approaching behavior, which was learned at the previous session. Animals showed a significant improvement in performance during the second half of this session (59.2±8.1% correct trials) as compared with the first half (23.8±3.8%) (Wilcoxon, $z=2.52$, $P<0.05$). The mean percentage of correct trials (±standard error of the means (SEM)) for the rats of this group was 45.4±6.2%. Rats of the "performance" group had significantly more correct trials (76.1±3.7%) than the "acquisition" group animals (Mann-Whitney, $z=2.90$, $P<0.01$). There were no significant differences in the number of food-cup checks between the "performance" group (261±33) and the "acquisition" group (271±21) animals. Altogether this implies that the total distance traveled did not differ between the groups.

Fos expression in the retrosplenial cortex

The mean percentage of c-Fos immunopositive nuclei (out of the total number of cells) in the retrosplenial cortex of the "acquisition" group rats (23.6±2.3; mean±SEM) was significantly higher than in animals of the "performance" group (12.0±1.5) (Mann-Whitney, $z=2.78$, $P<0.01$). However, the rats of the "performance" group showed a significantly higher mean percentage of Fos-positive neurons with respect to the animals of the "control" group (3.6±0.6; Mann-Whitney, $z=2.89$, $P<0.01$). There were no differences in the density of Fos-positive cells between the left and the right hemisphere either in the control or in the experimental animals (Wilcoxon, $z=1.29$, $P=0.198$). Layer-specific distribution of Fos-positive neurons in the right hemisphere of the retrosplenial cortex is given in Table 1 (also see Fig. 1 for examples of Fos photomicrographs). The upper cortical layers (II–IV) contained significantly more Fos-positive neurons than the lower layers (V–VI) for all the groups (Wilcoxon, $z=2.20$; $z=2.37$; $z=2.20$ respectively, $P<0.05$). The mean

Table 1. The mean numbers±S.E.M. per 1 mm^2 of Fos-positive neurons in layers II–IV, layer V, and layer VI of the retrosplenial cortex

	Layers		
	II–IV	V	VI
Acquisition group	599.7±75.9	195.6±13.2	236.1±15.0
Performance group	396.5±55.8	120.7±14.1	134.8±16.6
Control group	131.2±32.9	22.8±6.4	32.2±11.8

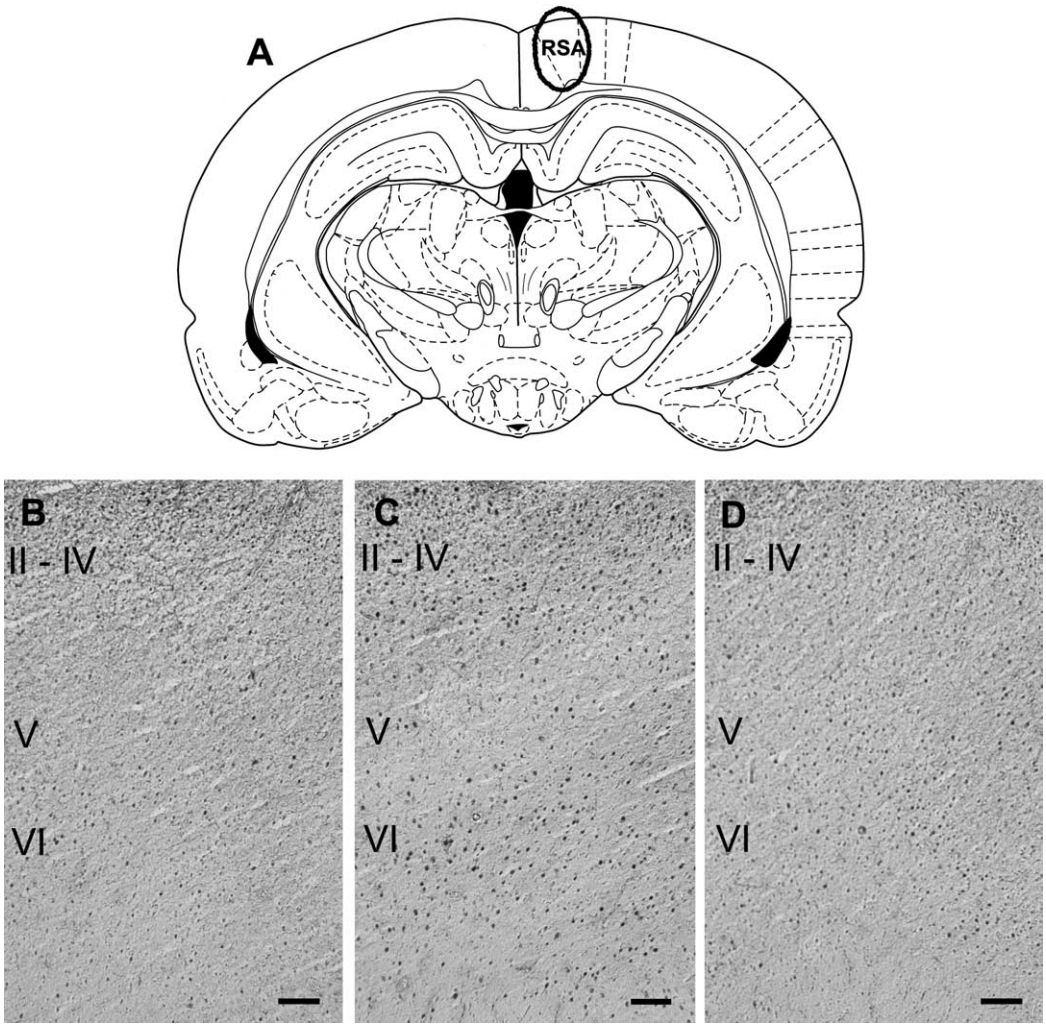

Fig. 1. Diagrams of coronal sections indicating a retrosplenial cortex (RSA) area sampled (A) and the photomicrographs showing Fos immunoreactive cells in the RSA of the control group (B), the acquisition group (C), the performance group (D) animals. The drawing (Paxinos and Watson, 1997) represents a coronal section 4.16 mm posterior to bregma. Scale bar=100 μm.

numbers of Fos-positive neurons in the layer V did not differ significantly from the layer VI for all the groups: acquisition, performance, and control (Wilcoxon, $z=1.18$, $z=0.11$, $z=0.31$ respectively; $P>0.05$).

Task-related neuronal activity in the retrosplenial cortex

Activity of 158 neurons was recorded from the retrosplenial cortex in six rats during the operant lever-press task. Fifty-eight neurons (36.7% of the recorded units) were classified as related to the SE-neurons (approach to the lever, lever-pressing, approach to the food-cup, taking of food from the food-cup) of the operant behavior, and 16 (10.1% of the recorded units) were classified as related to NSE-neurons. The retrosplenial cortex contained significantly more SE-neurons than NSE-neurons (χ^2 test, $\chi^2_{(1)}=23.8$, $P<0.001$). Activity of 84 neurons (53.2% of the recorded units) could not be functionally identified with respect to the behavior (UN-neurons).

Of the SE-neurons, activity of 24 units (15.2% of all recorded neurons) was related to the lever-press element of the task (Fig. 2).

Fos expression in the motor cortex

The mean percentage of c-Fos immunopositive neurons in the motor cortex did not differ significantly between the experimental groups: the “acquisition” group (3.6±1.6; mean±SEM.), the “performance” group (5.2±1.9), and the “control” group (2.6±0.4) (Kruskal-Wallis ANOVA, $\chi^2=2.08$; $df=2$; $P=0.556$). Furthermore, no differences were found in the number of Fos-positive cells between the left and right hemispheres of the control or experimental animals (Wilcoxon, $z=1.29$, $P=0.198$). Layer-specific distribution of Fos-positive neurons in the motor cortex of the right hemisphere is given in Table 2 (see also Fig. 3 for Fos photomicrographs). The groups did not differ in the number of Fos-positive neurons in layers II-IV, layer V, and layer VI (Kruskal-Wallis ANOVA, $\chi^2=2.06$, $df=2$; $\chi^2=1.41$, $df=2$;

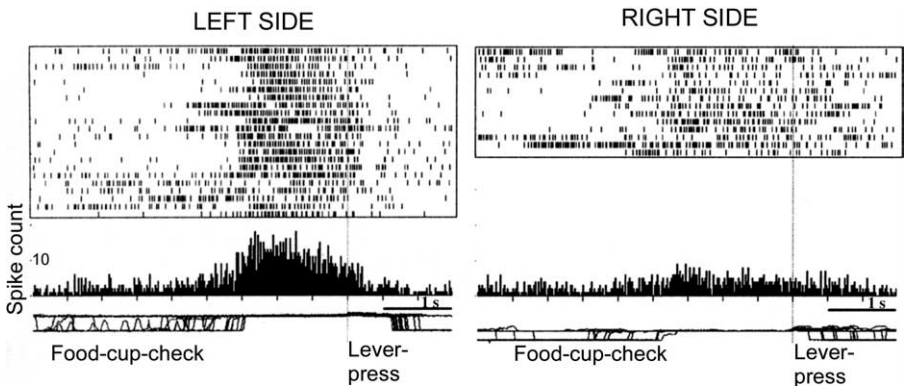

Fig. 2. An example of SE-neuron firing. The retrosplenial cortex cell exhibited activity relative to the approach to the left lever. The left panel represents the activity during an animal's performance along the left side of the box (22 trials). The right panel represents the activity during an animal's performance along the right side of the box (14 trials). The rasters are plotted against the lever-press (dashed line). The line below the rasters represents the superimposed behavioral acts.

χ^2=0.64, *df*=2 respectively, $P>0.05$). There was also no difference in the number of Fos-positive cells between the layers within the "acquisition" group (Friedman ANOVA, χ^2=2.89, *df*=2, P=0.236), the "performance" group (Friedman ANOVA, χ^2=3.63, *df*=2, P=0.163), and the control group (Friedman ANOVA, χ^2=2.17, *df*=2, P=0.337).

Task-related neuronal activity in the motor cortex

Activity of 105 neurons was recorded from the motor cortex in four rats during the operant lever-press task. Only five of these neurons (4.8% of the recorded units) were related to the SE-neurons, and 41 (39% of the recorded units) were classified as related to NSE-neurons (Fig. 4). The motor cortex contained significantly more NSE-neurons than SE-neurons (χ^2 test, $\chi^2_{(1)}$=28.2, $P<0.001$). Fifty-nine neurons (56.1% of the recorded units) were functionally unidentified (UN-neurons).

Of the SE-neurons, activity of only two units (1.9% of all recorded neurons) was related to the lever-press element of the task.

Comparison of Fos expression and number of task-related neurons in the retrosplenial and the motor cortex

The "acquisition" group contained significantly more Fos-positive cells (out of the total number of cells in a standard sampling area) in the retrosplenial cortex than in the motor cortex (Wilcoxon, z=2.37, $P<0.05$) (Fig. 5). There was no difference in the percentage of Fos-positive cells between the retrosplenial and the motor cortex in the "performance" group animals (Wilcoxon, z=1.57, P=0.116) as well as in the "control" group animals (Wilcoxon, z=1.47, P=0.141).

Table 2. The mean numbers±S.E.M. per 1 mm^2 of Fos-positive neurons in layers II–IV, layer V, and layer VI of the motor cortex

	Layers		
	II–IV	V	VI
Acquisition group	12.4±5.4	9.3±2.9	20.1±6.9
Performance group	37.1±18.9	21.7±9.4	38.4±16.6
Control group	13.3±4.4	9.5±2.7	19.0±5.4

The retrosplenial cortex of trained rats contained significantly more SE-neurons than the motor cortex (χ^2 test, $\chi^2_{(1)}$=49.2, $P<0.01$). The proportion of neurons that showed specific changes of firing in relation to the lever-pressing element of behavior was also significantly higher in the retrosplenial cortex than in the motor cortex (χ^2 test, $\chi^2_{(1)}$=11.1, $P<0.01$) (Fig. 6).

Thus, the retrosplenial cortex had significantly higher c-Fos expression compared with the motor cortex during acquisition of the lever-pressing operant task and contained significantly more neurons with lever-press-related activity in the trained rats.

To compare the results of the neuronal recording experiments to the c-Fos expression data, the number of lever-press related neurons out of the total number of cells was estimated by a method of representative cylinders (Henze et al., 2000) for the rat retrosplenial and the motor cortex. Assuming the retrosplenial cortex thickness of 1.5 mm (2 mm for the motor cortex) (Paxinos and Watson, 1997) and a cylinder radius of 50 μm for effective single unit extracellular recording (Mountcastle et al., 1957; Favorov and Whitsel, 1988), a single electrode can record spikes from cells in a cylinder with a volume of 11.78×10^{-3} mm^3 for the rat retrosplenial cortex and 15.70×10^{-3} mm^3 for the motor cortex. This volume was calculated to contain 598±40 cells in the retrosplenial cortex (635±23 in the motor cortex) based on an estimate of the retrosplenial cortex cell density of 50,800±1525/mm^3 (40,425±512/mm^3 for the motor cortex) taken from Nissl-stained sections. Given the 7.6±1.1 mean number of recorded cells-per-track (n=58) in the retrosplenial cortex in our experiments (11.5±0.6 in the motor cortex, n=27), it was estimated that 1.27% of all retrosplenial cortex cells and 1.81% of the motor cortex cell were active during rat behavior in the experimental chamber situation. Thus of the total number of cells in the sample area of the retrosplenial cortex 0.19% (15.2% of recorded neurons) had their activity related to the lever-pressing element of the

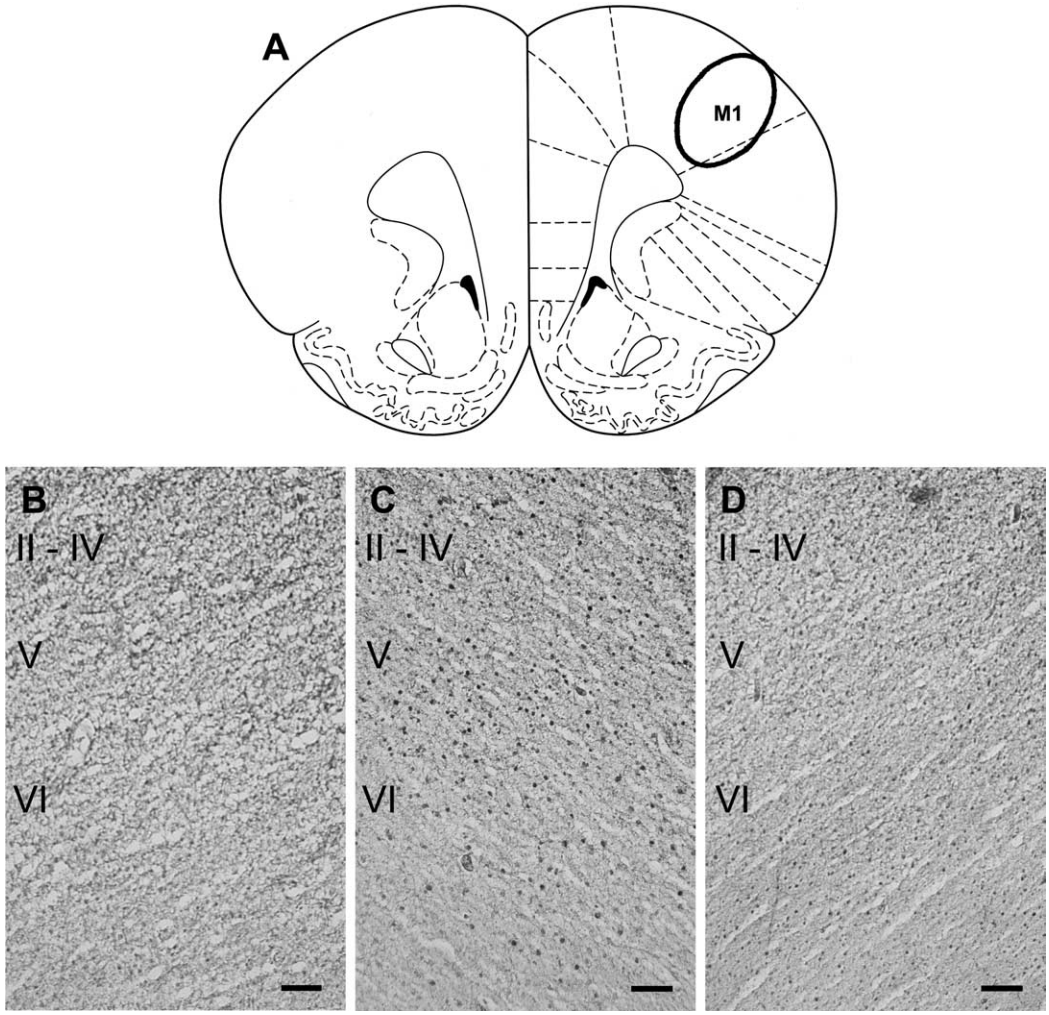

Fig. 3. Diagrams of coronal sections indicating a primary motor cortex (M1) area sampled (A) and the photomicrographs showing Fos immunoreactive cells in the motor cortex of the control group (B), the acquisition group (C), the performance group (D) animals. The drawing (Paxinos and Watson, 1997) represents a coronal section 3.2 mm anterior to bregma. Scale bar=100 μm.

behavior, while only about 0.03% (1.9% of recorded neurons) of the motor cortex neurons showed such task-related activity. The ratio of lever-press-related neurons in the retrosplenial cortex compared with the motor cortex neurons of the trained rats (6.3) was about the same as the ratio of c-Fos positive neurons in these two cortical areas during the acquisition of this task (7.1). However, a substantially higher absolute number of neurons demonstrated c-Fos activation in both cortical areas during initial acquisition of the lever-pressing behavior than was detected by electrophysiological recordings of task-related neurons in the trained rats.

DISCUSSION

Our experiments demonstrated that rats which acquired new operant appetitive behavior showed elevated c-Fos expression in the cerebral cortex as compared with the home cage control animals. The “acquisition” group animals displayed two types of behavior during the last experimental session. Initially, they demonstrated behavioral cycles consisting of level-approach followed by food-cup check that were learned during previous days and were not reinforced in the current session. Gradually, the animals acquired food-reinforced lever-pressing behavior, which progressively increased through the training session. Animals of the “performance” group had the statistically equal number of “food-cup check – lever approach – food-cup check” cycles which indicates that overall their locomotor activity was similar. However, c-Fos expression in the “acquisition” group was statistically higher indicating that it cannot be attributed to locomotor activity alone. Other studies also supported such conclusion that c-Fos expression is rather specifically related to acquisition of new experience than to locomotor activity alone or frustration, for example (Anokhin and Rose, 1991; Anokhin et al., 1991; Kleim et al., 1996; Suge and McCabe, 2004).

The main aim of the present study was to test whether the long-term changes in neuronal firing produced by instrumental learning occur in the same areas of the rat cerebral cortex where ITF c-Fos is substantially activated

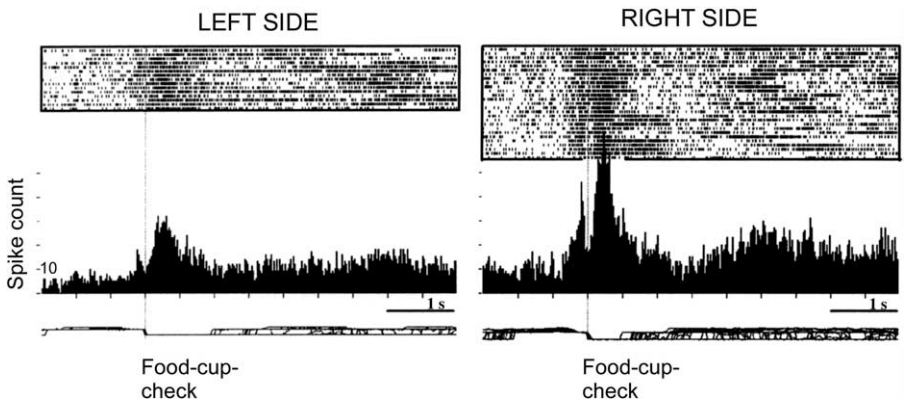

Fig. 4. An example of NSE-neuron firing. The motor cortex cell exhibited activity relative to the food-seizure from either food-cup. The left panel represents the activity during an animal's performance along the left side of the box (15 trials). The right panel represents the activity during an animal's performance along the right side of the box (27 trials). The rasters are plotted against the food-cup-check (dashed line). The line below the rasters represents the superimposed behavioral acts.

during acquisition of the same task. Our experiments demonstrated significantly higher number of Fos-positive cells and significantly higher number of neurons related to the specific elements of the task in the retrosplenial cortex of rats in contrast to the motor cortex. Thus, regularity of learning-induced c-Fos expression in this task resembled the regularity for the long-term changes in the learning-related neuronal activity in the two areas of the rat cerebral cortex.

Co-localization of learning-induced expression of ITFs and long-term neurophysiological changes is also supported by other studies. During the visual paired-associate learning in monkeys expression of ITF Zif268 has been most evident in the anterior temporal cortex (area 36) (Okuno and Miyashita, 1996), that is in the field which contained the neurons with specific activity related to the presentation of paired associates (Sakai and Miyashita, 1991). Studies on neuronal activity in the rodent orbitofrontal cortex (Schoenbaum and Eichenbaum, 1995; Schoenbaum et al., 1999) and basolateral amygdala (Muramoto et al., 1993; Quirk et al., 1995; Schoenbaum et al., 1999) have reported that neurons in these regions encode associative information. These regions have also showed learning-specific increase of c-Fos immunoreactivity after odor-reward associative task (Tronel and Sara, 2002). In the conditioned taste aversion task the nucleus of the solitary tract has been demonstrated to contain neurons with specific activity related to the presentation of the conditioned taste (Chang and Scott, 1984). Induction of c-Fos expression was prominent in the same region in the conditioned animals (Swank and Bernstein, 1994; Swank et al., 1995; Swank, 2000). Furthermore, injection of c-Fos antisense into this structure has been shown to block the acquisition of the conditioned taste aversion (Swank et al., 1996), which points to causal links between ITF expression, long-term neuronal changes and learning.

However, the above studies supplied little information on distribution of task-related neurons in the regions that did not show learning-induced increase in ITFs expression. In order to provide such negative control in this study, we additionally examined the motor cortex of the trained rats. The anterolateral area of the motor cortex has been shown to contain a very low number of task-specific neurons in rabbits (Alexandrov et al., 1991), and it was expected that a similar low number would be found for the lever-press operant behavior in rats. Indeed, the rat motor cortex con-

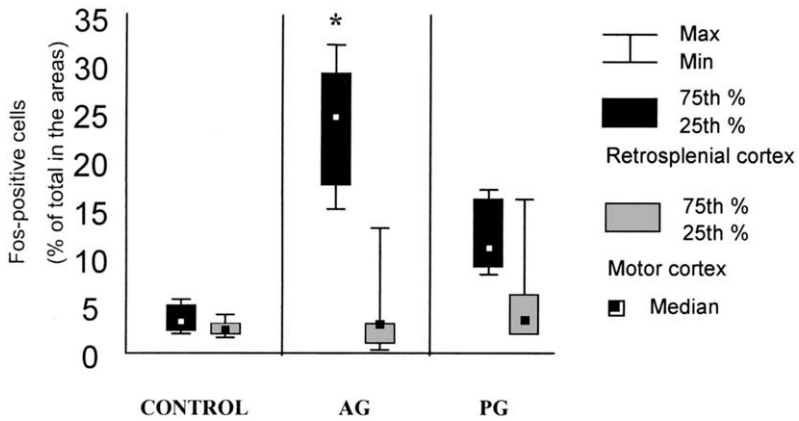

Fig. 5. The percent of Fos-positive neurons in the retrosplenial cortex and in the motor cortex of the control group, the acquisition group (AG), and the performance group (PG) animals. * $P<0.05$, compared with the motor cortex.

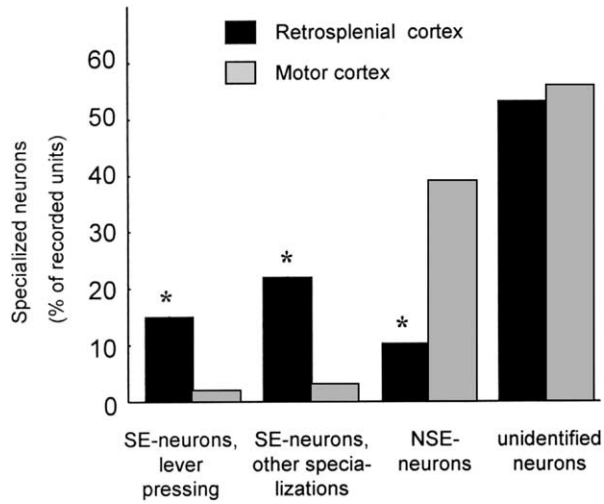

Fig. 6. The percent of neurons with different specializations in the retrosplenial and the motor cortex. * $P<0.01$, compared with the motor cortex.

tained much fewer task-specific lever-press neurons than the retrosplenial cortex. It also did not show any significant increase in c-Fos expression after instrumental lever-press learning. Thus, of the two cortical areas, only the retrosplenial cortex, which had significant learning-induced c-Fos expression, contained a substantial number of neurons with task-specific activity. These data, together with those presented above, suggest that learning-induced c-Fos expression and specialization of neuronal activity with respect to the acquired task might be closely related.

The detailed relationship between learning-induced expression of c-Fos and long-term changes of neuronal activity related to the learned behavior is beyond the scope of the present experimental study. However, the cascade of cellular events associated with learning involves a number of components that can bridge ITFs activation with long-term changes in the neuronal activity. Molecular events that underlie long-term memory formation include activation of NMDA (N-methyl-D-aspartate) receptors, an increase in intracellular Ca^{2+}, activation of protein kinases and neuronal transcriptional cascades started with ITFs expression (Abel et al., 1997; Abel and Lattal, 2001; Kandel, 2001). Several of these intracellular signaling events have been recently shown to be involved in long-term stability of hippocampal place cells whose activity is restricted to part of a given learned space (Rotenberg et al., 1996, 2000; Kentros et al., 1998; Agnihotri et al., 2004). Instability of place cell firing fields has been observed with decreased protein kinase A activity (Rotenberg et al., 2000) and αCaMK II activity (Rotenberg et al., 1996) in transgenic mice. Moreover, both of these mouse strains had impaired long-term spatial memory (Mayford et al., 1996; Abel et al., 1997). Also, instability of place cell firing fields was shown under NMDA receptor blockade (Kentros et al., 1998) and under protein synthesis inhibition (Agnihotri et al., 2004). A significant decrease in the spatial specificity of individual place fields has been also found in mice with a CA1 pyramidal cell-specific knockout of the NMDAR1 gene (McHugh et al., 1996). These data suggest that the cellular signaling events that result in ITFs expression might be causally related to formation of functional firing patterns in neurons. The correspondence between Arc expression, one of the immediate early genes, and neuronal activation related to particular behavioral correlates has been also suggested earlier (Guzowski et al., 1999; Vazdarajanova and Guzowski, 2004; Burke et al., 2005).

Our study showed that at the time of learning the lever-pressing behavior, the percentage of Fos-positive cells in the retrosplenial cortex (23.6±2.3% mean±SEM.) and in the motor cortex (3.6±1.6%) substantially exceeded the estimated percentage of lever-press-related neurons in these areas of the well-trained rats (0.19% and 0.03%, respectively).

The data regarding the low number of task-related neurons suggest cellular sparsity in encoding memories, which is consistent with prior observations (Barnes et al., 1990; Wilson and McNaughton, 1993). Discrepancy between the number of Fos-positive neurons and task-related neurons might be due to the long time period over which c-Fos expression is accumulated between the beginning of training and animals' decapitation at 75 min after the end of the experimental session. Post-acquisition consolidation processes shown in experiments with temporal inactivation of different structures at different times (Sacchetti et al., 1999) and experience-dependent neuronal reverberation shown by simultaneous neuronal ensemble recordings (Hoffman and McNaughton, 2002; Ribeiro and Nicolelis, 2004) might all contribute to the pattern of c-Fos expression observed 75 minutes after the training. Another possibility is that only a small number of neurons compared with those, initially active in learning, form and retain task-specific firing patterns in the well-trained animals. c-Fos expression has been reported to precede the increase in synapse number in the motor cortex after motor skill learning for at least one day (Kleim et al., 1996) and might be only one of the components of the transcriptional activation of a neuron resulting in acquisition of its new phenotype (Kaczmarek and Kaminska, 1989) or "memory of the following events" (Clayton, 2000), which is revealed by task-specific neuronal firing. Therefore, c-Fos activation in an abundant number of neurons during learning might only prime the subsequent long-term changes and form a background for the following selection of specific neuronal groups as proposed by the selection theories of learning (Shvyrkov, 1986; Edelman, 1989).

CONCLUSION

In summary, the present study demonstrated that c-Fos induction during learning of lever-press operant task is high in the rat retrosplenial cortex, which is also characterized by the presence of neurons with specific electrophysiological activity related to the acquired behavior. Conversely, the motor cortex, which contains significantly lower numbers of such task-specific neurons, does not show an increased level of c-Fos expression after the

instrumental learning. These data support the hypothesis that learning-induced changes in c-Fos gene expression might underlie formation of specific neuronal activity, which maintains the acquired new behavior.

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