

EXPLORATORY ACTIVITY AND A CONDITIONED FEAR RESPONSE: CORRELATION WITH CORTICAL AND SUBCORTICAL BINDING OF THE $\alpha 4\beta 2$ NICOTINIC RECEPTOR AGONIST [^3H]-EPIBATIDINE

*Halina Sienkiewicz-Jarosz¹, Piotr Maciejak², Andrzej Bidziński²,
Janusz Szynkler³, Marek Siemiątkowski⁴, Agnieszka Członkowska³,
Małgorzata Lehner², Adam Płaźnik^{2,3,#}*

¹Department of Neurology, ²Department of Neurochemistry, ⁴Department of Pharmacology and Physiology
of the Nervous System, Institute of Psychiatry and Neurology, Sobieskiego 9, PL 02-957 Warszawa,

³Department of Experimental and Clinical Pharmacology, Medical University, Krakowskie Przedmieście 26/28,
PL 00-927 Warszawa, Poland

*Exploratory activity and a conditioned fear response: correlation with
cortical and subcortical binding of the $\alpha 4\beta 2$ nicotinic receptor agonist [^3H]-
epibatidine. H. SIENKIEWICZ-JAROSZ, P. MACIEJAK, A. BIDZIŃSKI,
J. SZYNDLER, M. SIEMIĄTKOWSKI, A.I. CZŁONKOWSKA, M. LEH-
NER, A. PŁAŻNIK. Pol. J. Pharmacol., 2003, 55, 17–23.*

Rat behavior in the open field and the conditioned fear response test was correlated with specific binding of the $\alpha 4\beta 2$ nicotinic acetylcholine receptor (nAChR) agonist, [^3H]-epibatidine, assayed in different brain structures by autoradiography. A significant positive correlation was found between the ligand binding in the frontal cortex ($r = 0.529$, $p < 0.011$), the entorhinal cortex ($r = 0.603$, $p < 0.003$), and CA1 layer of the hippocampus ($r = 0.465$, $p < 0.029$), and the conditioned freezing reaction in the contextual fear conditioning test. In the frontal cortex, there was also a significant positive correlation between [^3H]-epibatidine binding and preconditioned freezing reaction ($r = 0.469$, $p < 0.028$), and a negative correlation with rat motility ($r = -0.452$, $p < 0.035$). Rat motor activity correlated in a negative way with preconditioned freezing reaction ($r = -0.436$, $p < 0.043$), and in a positive way with the number of entries into the central sector of the open field ($r = 0.690$, $p < 0.001$). The neophobia-related parameter of the open field behavior (the number of central entries) did not correlate with the [^3H]-epibatidine binding. Factor analysis confirmed these findings and showed that rat behavioral parameters measured in the tests of neophobia and conditioned freezing were loading on different factors, thus, pointing to separate central mechanisms operating in both behavioral models of anxiety. Furthermore, factor analysis showed that rat conditioned freezing behavior and [^3H]-epibatidine binding in the CA1 layer of the hippocampus and entorhinal cortex, represented similar central processes.

These findings suggest that rat emotional reactions evoked by different stressors (neophobia vs. conditioned fear) are differently regulated by the central cholinergic system. The presented data indicate also a significant, structure dependent correlation between rat conditioned emotional reaction and the $\alpha 4\beta 2$ nAChR ligand binding, in the rat cortical forebrain structures.

Key words: [^3H]-epibatidine, autoradiography, exploratory behavior, freezing reaction, rats

INTRODUCTION

The contribution of the nicotinic receptor family in different brain structures to the regulation of various behavioral processes, has not yet been clarified enough [4, 5, 14, 15, 18]. Nicotine, a non-selective nicotinic acetylcholine receptor (nAChR) ligand, has cognitive and memory-enhancing properties, and has been reported to reduce anxiety in humans and in animal models of anxiety [7, 9, 13, 14, 17]. Recently, the preferential $\alpha 4\beta 2$ nAChR subunit antagonist, dihydro- β -erythroidine hydrobromide, was found to antagonize the anxiolytic effect of nicotine in the social interaction test in rats, following its intra-dorsal raphe infusion [3]. These data indicate that the anxiolytic effect of nicotine may be mediated by the $\alpha 4\beta 2$ nAChR receptor subtype.

The aim of the present study was to further explore this problem, by examining the correlation of rat motor and exploratory reactions, and a conditioned fear response, with the binding of the nicotinic receptor agonist [^3H]-epibatidine, a radioligand with exceptionally high binding affinity for $\alpha 4\beta 2$ nAChRs [1, 4, 12, 15], in several forebrain cortical structures, visualized by quantitative receptor autoradiography. The behavioral parameters for the study were selected on the grounds of recent findings, indicating a selective contribution of nicotinic receptor ligands to the regulation of emotional processes [4, 7, 8]. The analysis was concentrated on the brain cortical structures and hippocampus involved in the processes of cognition and learning, and the basal ganglia contributing to motor activity regulation. In this way, it was possible to differentiate the cognitive and motor components of a cholinergic regulation of rat behavior. Such analysis could provide us also with new information about the role that the subtypes of nicotinic receptors play in different brain structures, in the organization and expression of emotional behavior.

Although it could be considered an oversimplification to look for a direct relationship between a single receptor population and a complex behavioral pattern, the literature data indicate that such studies could provide us with new information, especially when performed in specific brain structures. For example, using similar approach, we recently found a highly significant, structure-dependent correlation between rat motor behavior

and the dopamine D_1 receptor ligand binding ([^3H]-SCH23390) within the nigrostriatal system [16]. No correlation was revealed between the ligand binding in the examined brain areas and the freezing reaction in the contextual fear conditioning test. The presented data indicated for the first time a selective contribution of the dopamine D_1 receptors in the examined brain structures to the modulation of rat motor behavior, and the absence of a simple relationship between individually variable reaction to the stressful event and the dopamine D_1 receptor ligand binding in the rat brain.

MATERIALS and METHODS

Animals

Male Wistar rats (200 ± 20 g), bought from a licensed breeder, were housed in standard laboratory conditions under a 12-h light/dark cycle (lights on at 6 a.m.), at a constant temperature ($21 \pm 2^\circ\text{C}$) and 70% humidity. All experimental procedures using animal subjects were approved by the Committee for Animal Care and Use at the Medical University, Warszawa.

Behavioral studies

Open field test

The test was performed in a soundproof chamber under dim light and continuous white noise (65 dB). The open field apparatus consisted of two round arenas (80 cm in diameter) with 30 cm high walls. Locomotor activity of naive rats, and the number of entries into the central sector of the open field (50 cm in diameter), were recorded and analyzed with the PC-based Videomot System (TSE, Bad Homburg, Germany). Two weeks later the rats were examined in the contextual fear conditioning test.

Contextual fear conditioning test

The test was carried out in two boxes ($30 \times 30 \times 60$ cm each) made of Plexiglas, with a grid floor made of stainless steel bars wired to shock generator. The boxes were cleaned after each trial with 95% ethanol. The experiment was performed during three consecutive days in the same testing boxes and experimental chamber. On the first day, the animals were placed separately for 2 min in a training box, for adaptation to the experimental conditions. On the following day, after the animals

had been placed in the experimental box, they were observed and videotaped for five min, *via* a short-circuit television, for spontaneously occurring freezing behavior (baseline freezing). Immediately afterwards, the animals received three 0.5 s foot shocks (trains of stimuli: 0.7 mA, 150 ms, repeated every 60 s). The animals were removed from the testing boxes 3 min after the last shock was delivered. On the following day, the freezing behavior of rats was examined for 10 min. The conditioned response was recorded with the help of a video camera in order to analyze the freezing reaction. The freezing behavior was defined as the absence of any visible body movements except for those required for respiration.

Autoradiography

A detailed description of the method for receptor autoradiography has been published earlier [10]. Briefly, one week after the last behavioral experiment the rats were sacrificed, their brains were rapidly removed, frozen in isopentane (-30 to -40°C) cooled with dry ice and stored at -70°C . Coronal sections were cut at $12\ \mu\text{m}$ slices on a microtome at -20°C and thaw mounted onto gelatin-coated glass slides. The slides were incubated in 50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl, 2.5 mM CaCl_2 , 1 mM MgCl_2 (pH-7.0) containing 0.5 nM [^3H]-epibatidine (40 Ci/mmol, Amersham) for 40 min at room temperature. Non-specific binding was determined in the additional presence of 300 μM nicotine. The sections were then washed 2×5 min in the ice-cold buffer and dipped in distilled water. The slides were dried under a stream of nitrogen, placed in X-ray cassettes and exposed to [^3H] Hyperfilm (Amersham) at 4°C . After exposure for 4 months the films were developed with a Kodak LX-24 developer for 5–7 min, fixed, washed and dried. Quantitative analysis of the autoradiogram was performed with an image analysis system (Imaging Research Inc., St. Catharines, Canada). Non-specific binding of [^3H]-epibatidine was negligible.

Statistical analysis

The data are shown as means \pm SDM. The results were analyzed using Pearson r correlation with case-wise deletion of missing data. The results of behavioral and biochemical tests were analyzed also by factor analysis, using a principal components solution (PCA) with an orthogonal rotation (varimax) of the factor matrix, which ensures that

the extracted factors are independent of one another and should, therefore, reflect separate processes. The number of factors extracted for the analysis was selected using a combination of two criteria: the 75% variance rule (the relevant matrix variance is accounted for when the sum of the proportionate contributions of the eigenvalues exceeds 0.75), and the root curve analysis (the point of inflection of a plot of the eigenvalues from largest to smallest). The factors for eigenvalues > 1.0 are presented left to right in an order that corresponds to the decreasing size of the proportion of the original variance represented by each factor. The contribution of each behavioral and biochemical variable to each factor is referred to as a factor loading. The higher the loading, the better the variable reflects a particular factor. Only factors with loading higher than 0.7 (or lower than -0.7) were considered significant. The statistical package Statistica for Windows, Release 6 (StatSoft Inc., USA), was used for statistical calculations. The confidence limit of $p < 0.05$ was considered statistically significant.

RESULTS

Rat behavioral parameters in the open field and the contextual fear conditioning tests (summarized in Table 1) were correlated with the specific bind-

Table 1. The behavioral parameters measured in the open field and contextual fear conditioning test, and the specific binding of [^3H]-epibatidine within different brain structures (nCi/mg of tissue)

Correlated parameters	n	Mean \pm SDM
Motor activity (cm)	23	3055.34 \pm 1177.10
Number of entries	23	4.35 \pm 4.44
Freezing time (baseline) (s)	24	13.96 \pm 12.52
Conditioned freezing time (s)	24	205.68 \pm 121.94
[^3H]-epibatidine binding in:		
Frontal cortex	24	0.83 \pm 0.18
Entorhinal cortex	24	0.58 \pm 0.14
Hippocampus (CA1)	24	0.27 \pm 0.07
Septum	23	0.70 \pm 0.18
Caudate putamen	23	1.14 \pm 0.17

The conditioned freezing time was obtained by correction of freezing reaction in the test with the baseline level (baseline freezing time) observed during first 5 min of the pretest. The data are shown as means \pm SDM; n = number of rats

Table 2. Correlations between behavioral parameters and [³H]-epibatidine binding to different brain structures

Correlated parameters	Pearson's coefficient	p
FRCX/LOC	(-) 0.452	0.035
FRCX/ENT	(-) 0.274	0.218
FRCX/BFREE	(+) 0.469	0.028
FRCX/CFREE	(+) 0.529	0.011
ECX/LOC	(-) 0.105	0.642
ECX/ENT	(+) 0.071	0.754
ECX/BFREE	(+) 0.274	0.217
ECX/CFREE	(+) 0.603	0.003
CA1/LOC	(+) 0.153	0.497
CA1/ENT	(+) 0.241	0.280
CA1/BFREE	(+) 0.020	0.931
CA1/CFREE	(+) 0.465	0.029
SEPTUM/LOC	(+) 0.095	0.674
SEPTUM/ENT	(+) 0.009	0.969
SEPTUM/BFREE	(+) 0.078	0.731
SEPTUM/CFREE	(+) 0.352	0.108
CPU/LOC	(-) 0.218	0.329
CPU/ENT	(-) 0.181	0.420
CPU/BFREE	(-) 0.060	0.791
CPU/CFREE	(+) 0.159	0.481
LOC/ENT	(+) 0.690	0.001
LOC/BFREE	(-) 0.436	0.043
LOC/CFREE	(-) 0.201	0.371

FRX – frontal cortex; ECX – entorhinal cortex; CA1 – hippocampus, layer CA1; Septum – medial septal nuclei; CPU – caudate putamen; LOC – locomotor activity; ENT – entries into the central part of the open field; BFREE – baseline freezing; CFREE – conditioned freezing

ing of nicotinic receptor agonist [³H]-epibatidine in different cortical and subcortical brain structures (Table 2). A significant positive correlation was found between the ligand binding in the frontal cortex ($r = 0.529$, $p < 0.011$), the entorhinal cortex ($r = 0.603$, $p < 0.003$), and CA1 layer of the hippocampus ($r = 0.465$, $p < 0.029$), and conditioned freezing reaction in the contextual fear conditioning test. In the frontal cortex, there was also a significant positive correlation between [³H]-epibatidine binding and baseline, preconditioned freezing reaction of rats ($r = 0.469$, $p < 0.028$), and a negative correlation with rat motility ($r = -0.452$, $p < 0.035$). Rat motor activity correlated in a negative way with conditioned freezing reaction ($r = -0.436$, $p < 0.043$), and in a positive way with the number of entries into the central sector of the open field ($r = 0.690$, $p < 0.001$). The maximum and minimum values of specific binding of [³H]-epibatidine to the forebrain cortical structures are shown in Figure 1.

Factor analysis of behavioral and epibatidine binding data allowed for extraction of several independent factors (Tab. 3). Four factors with eigenvalues > 1 representing 83.54% of the total variance emerged: Factor 1 (eigenvalue = 6.550) represented the parameter measured in the central area of the open field test (the number of central entries), Factor 2 (eigenvalue = 3.836) with high loading of the parameter measured in the conditioned freezing test (total time of conditioned freezing behavior), and epibatidine binding in the CA1 layer of the hippocampus, and in the entorhinal cortex, Factor 3 (eigenvalue = 2.486) representing the binding of epibatidine in the septum and nucleus caudatus, and Factor 4 (eigenvalue = 1.331) with

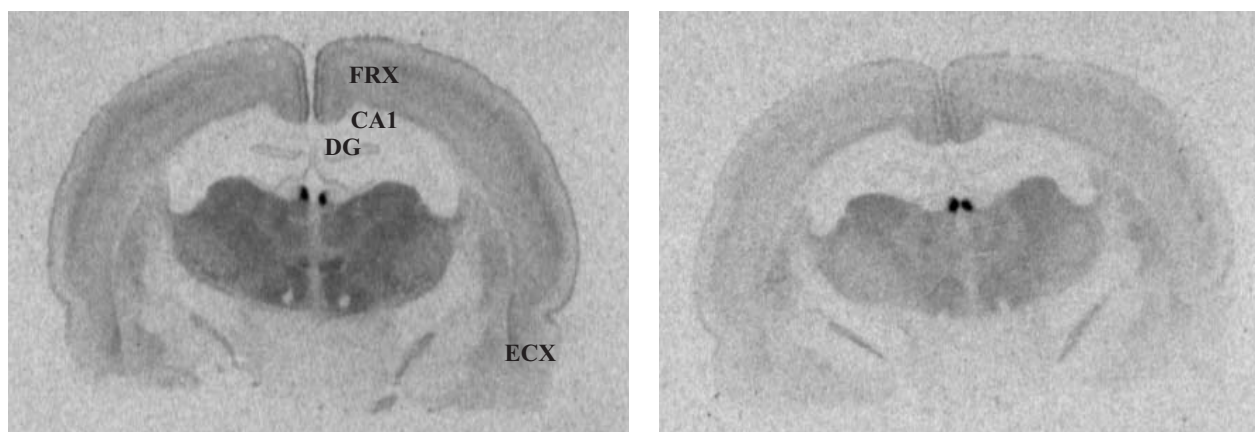


Fig. 1. Autoradiograms illustrating the maximum (left) and minimum (right) specific binding of [³H]-epibatidine to brain structures. All other abbreviations as in Table 2

Table 3. Factor analysis. See Results section for description. * = loadings higher than 0.7 (or lower than -0.7)

An analyzed parameters Eigenvalue (% of total variance)	Factor 1 6.550 (38.53)	Factor 2 3.836 (22.56)	Factor 3 2.486 (14.62)	Factor 4 1.331 (7.83)
Total distance	0.623	-0.001	-0.001	-0.685
Entries	0.954*	0.016	-0.039	-0.221
Basal freezing	-0.191	0.304	-0.070	0.692
Total freezing (1–10 min)	-0.146	0.903*	0.149	0.222
[³H]-epibatidine binding:				
CA1 area of hippocampus	0.220	0.772*	-0.157	-0.188
Septum	0.008	0.322	0.814*	-0.146
Entorhinal cortex	0.137	0.748*	0.289	0.139
Frontal cortex	-0.099	0.281	0.595	0.639
Caudate putamen	-0.134	0.003	-0.911*	0.083

close to significance level loading of motor activity in the open field test.

DISCUSSION

The main result of the present study is a striking correlation between specific binding of the $\alpha 4\beta 2$ nAChR ligand in the forebrain cortical structures and rat freezing reaction. [³H]-epibatidine binding in the frontal cortex, the entorhinal cortex and CA1 layer of the hippocampus correlated in a positive way with the conditioned, fear-related response. These data significantly contribute to the previously published reports on the possible neuroanatomical representation of the nicotinic receptor-related functions [6–8, 15].

Epibatidine has exceptionally high binding affinity for $\alpha 4\beta 2$ nAChRs ($K_i = 19$ pM) [1, 12, 14, 15]. Binding to $\alpha 7$ nAChRs is about 10,000-times lower than to $\alpha 4\beta 2$ nAChRs ($K_i \sim 200$ nM). Acetylcholine and nicotine bind to the $\alpha 4\beta 2$ nAChRs with much lower affinity ($K_i = 1–11$ nM) [1, 6, 14]. Thus, [³H]-epibatidine has been proved to be a very useful radioligand for monitoring $\alpha 4\beta 2$ nAChRs in the brain structures.

In the brain, the most abundant nAChR subtype is the $\alpha 4\beta 2$ nAChR [18]. High affinity binding sites for nicotine and epibatidine were detected in the thalamus, hippocampus, substantia nigra, cerebral cortex and cerebellum [6, 14, 18]. The cholinergic septo-hippocampal system, and closely anatomically and functionally related entorhinal cortex, rich in the $\alpha 4\beta 2$ nAChRs, have been implicated in

the control of anxiety, and these structures have been proposed to be an important site for the cholinergic vs. GABAergic interaction [8, 11]. Nicotine has also been shown to interact directly with the hippocampal cholinergic system to produce anxiolytic effect [7, 8, 15]. The presented data indicate that the $\alpha 4\beta 2$ nAChR subtype in the cortical forebrain structures may significantly contribute to the expression of emotional behavior. Accordingly, the aged male knock-out mice, lacking the $\beta 2$ subunit of the nicotinic receptor, showed impaired freezing reaction to the aversively conditioned both context and tone, in comparison with aged wild type males [2]. Recently, the preferential $\alpha 4\beta 2$ nAChR subunit antagonist, dihydro- β -erythroidine hydrobromide, was found to antagonize the anxiolytic effect of nicotine following intra-dorsal raphe infusion, in the social interaction test in rats [3].

All these data indicate a direct involvement of the $\alpha 4\beta 2$ nAChRs in the control of emotional processes. Our findings strongly support such corollary. Individually variable and genetically determined expression of the $\alpha 4\beta 2$ nAChR subtype (labelled with [³H]-epibatidine) in the frontal cortex, the entorhinal cortex and CA1 layer of the hippocampus, significantly correlated with the conditioned fear-related response in rats. The most striking correlation ($r = (+) 0.603$) appeared between the binding of [³H]-epibatidine in the entorhinal cortex and the conditioned freezing reaction. This structure belongs to the neural circuitry transmitting information from the frontal cortex to the amygdala. However, it is difficult to interpret the positive di-

rection of this correlation without further studies, especially as nicotine was found to exert both anxiolytic and anxiogenic effects dependently on the behavioral model of anxiety and the way of administration (peripheral vs. intrahippocampal injections) [7–9, 17].

Interestingly, the neophobia-related parameter of the open field behavior (the number of central entries) did not correlate with the [³H]-epibatidine binding. This finding indicates that rat emotional reactions controlled by different stressors (neophobia vs. conditioned fear) are differently regulated by the central cholinergic system. It was also found that [³H]-epibatidine binding in the frontal cortex correlated negatively with rat motor activity, and in a positive way with conditioned freezing reaction. This means that nicotinic receptors in the frontal cortex most probably are oppositely involved in the control of rat motor activity and aversively motivated conditioned behavior. As could be expected, rat motility was positively correlated with the number of entries into the central area of the open field, and inversely related to the duration of baseline, unconditioned freezing reaction.

Factor analysis confirmed such corollaries, and provided further evidence for the selective contribution of nicotinic receptors to the control of emotional behavior. It showed that rat behavior in the tests of neophobia and conditioned freezing were loading on different factors, thus pointing to separate central mechanisms operating in both behavioral models of anxiety. Furthermore, factor analysis indicated that rat conditioned freezing behavior and [³H]-epibatidine binding in the CA1 layer of the hippocampus and entorhinal cortex, are under control of similar central processes. Another factor reflected the binding of epibatidine in the septum and caudate-putamen.

Summing up, there is a growing body of evidence indicating that nicotinic receptors in the cortical forebrain structures may selectively participate in the regulation of emotional behavior. The present results clearly demonstrate for the first time an opposing and structure-dependent correlation between rat motor activity and the behavior controlled by a conditioned fear, and the $\alpha 4\beta 2$ nicotinic receptor ligand binding in the cortical forebrain structures. However, as they are mostly based on a correlative statistics, it would be necessary to support them by more behavioral results, obtained

with the help of other methods (e.g. pharmacological studies using selective nicotinic receptor agonists and antagonists).

Acknowledgments. The study was supported by a grant no. 4 P05A 009 18 from the State Committee for Scientific Research. H. Sienkiewicz-Jarosz, M. Siemiątkowski and A.I. Członkowska were also supported by grants from the Foundation for Polish Science.

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Received: September 30, 2002; in revised form: November 18, 2002.