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CONTENTS

EFFECT OF NIFEDIPINE ON ANALGESIA INDUCED BY GABA$_A$ AND GABA$_B$ RECEPTOR AGONISTS IN RATS

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Abstract

In the present study, we found that DHP calcium channel antagonist nifedipine exerted an antinociceptive activity following systemic administration. In agreement with earlier observations, these data suggest that nifedipine might possess a certain analgesic properties. In order to test a possibility that GABA-sensitive mechanisms may be involved in the antinociceptive effect of nifedipine, series of experiments were carried out in which the effects of subanalgesic dose of nifedipine on GABA$_A$ agonist muscimol- and GABA$_B$ agonist baclofen-induced analgesia in the hot-plate test in rats were studied. When co-administered with baclofen nifedipine significantly potentiated baclofen-induced analgesia. On the other hand, co-administration of nifedipine with muscimol did not affect muscimol-induced analgesia. Data obtained in our study suggest the existence of functional interaction between dihydropyridine binding site and GABA$_B$ receptors.

Introduction

Gamma-aminobutyric acid (GABA) receptors have been classified into GABA$_A$ and GABA$_B$ subtypes on the basis of their pharmacological and anatomical properties [4]. GABA$_A$ receptor contains a chloride channel, while GABA$_B$ receptors are thought to modulate potassium and calcium ion conductances [9]. GABAergic mechanisms appear to be involved in antinociceptive processes. Thus, it has been shown that both GABA$_A$ and GABA$_B$ receptor agonists can induce analgesia in variety of animal test systems when administrated either intraperitoneally [14, 16] or centrally [17].

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Current evidence indicates that calcium ion plays an important role in the nociception. It is known that the intracerebroventricular or the subcutaneous administration of calcium antagonizes analgesia whereas the administration of EDTA induces opposite effects [11]. Moreover, the analgesic action of opiates is accompanied by their interaction with calcium influx into the cells through the voltage-sensitive calcium channels [5]. These calcium channels are sensitive to blockade by dihydropyridine (DHP) antagonists and to activation by the DHP agonist BAY K8644 [19] suggesting that the functional role of central DHP binding sites might be to modulate the Ca^{2+} entry into the cells subsequently modifying the Ca^{2+}-dependent cellular functions. Del Pozo and co-workers recently found that calcium channel antagonists produce analgesic effect when administrated alone as well as enhance antinociceptive actions of morphine in rodents [6].

In view of the above findings the present study was designed to examine the effects of DHP calcium channel antagonist nifedipine on analgesia induced by $GABA_A$ and $GABA_B$ receptor agonists muscimol and baclofen. Finally, since it has been shown that some of the pharmacological effects of ethanol may be mediated through GABA receptors [1] we decided to study also the effects of DHP calcium channel antagonist and agonist on ethanol-induced analgesia.

Materials and method

Animals. The experiments were carried out on female Wistar rats, weighing 220-240 g, kept under standard laboratory conditions, with free access to standard laboratory food and tap water, at room temperature of approx 22' C, in natural day-night cycle. On the day of testing, subjects were transported to a laboratory room, weighed, and assigned randomly to receive either vehicle or drug treatment.

Drugs and drug treatment. Baclofen (CIBA-GEIGY, Switzerland) and muscimol (Biochemica Merck, Germany) were dissolved in distilled water and administered subcutaneously 30 min prior to test. Nifedipine (Orion Pharmaceutica, Finland) and BAY K8644 (Bayer, Germany) were suspended in distilled water, with 2-4 drops of Tween-80 and administered intraperitoneally 60 min and 15 min prior to test, respectively. Ethanol was administered intragastrally as 10% water solution 30 min prior to test. The solutions of nifedipine and BAY k8644 were always made ex tempore and protected against light by storing in dark bottles wrapped tightly in aluminum foil.

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Apparatus and procedures. The responsiveness of rats to painful stimulus was assessed using the hot plate test, [22, 23]. The plate surface temperature was maintained at 55.4° C and a cutoff time of 30 sec was imposed. Baseline testing was done prior to drug administration by recording the latency of response (licking of hind paws or jumping) in seconds. Animals were retested as for base-line testing after the drug administration. The analgesic data were expressed as "analgesic index" as described by Cox (1968). It is given by the formula $(L_{obs} - L_i)\backslash(L_{max} - L_i)100$ where L_{obs}, L_i and L_{max} are latencies (sec) after the treatment, initial latency and cutoff time respectively.

Statistical analysis. The data were analyzed with analysis of variance (ANOVA) followed, where appropriate, with Duncan's multiple range test between individual groups.

Results

The effect of nifedipine on nociceptive response of the animals in the hot-plate test is shown in Fig. 1. The dose-response curve for nifedipine revealed that this dihydropyridine calcium channel antagonist produced a significant analgesic effect ($F(5, 54) = 6.57$; $P = 0.0001$) in the doses 2.5-20 mg/kg. The lower dose of nifedipine tested (1.0 mg/kg) was ineffective. Muscimol (1 mg/kg s.c.), ethanol (2 g/kg i.g.) and baclofen (2.5-5 mg/kg s.c.) when administered alone produced analgesia in rats as it was evidenced by an increase in latency of analgesic response (Fig. 2-4). Nifedipine given in a low subanalgesic dose (1 mg/kg i.p.) significantly enhanced the analgesic response induced by 5.0 mg/kg baclofen (Fig. 2) but did not affect muscimol-induced analgesia (Fig. 3). Also, nifedipine (1 mg/kg i.p.) tended to increase analgesic response induced by ethanol (1-2 g/kg i.g.). However, the data were not significant (Fig. 4). Co-administration of a higher (analgesic) dose of nifedipine (10 mg/kg i.p.) with muscimol, baclofen and ethanol in the doses mentioned above (data not shown) resulted in an additive effect on nociceptive response latency.

Dihydropyridine calcium channel agonist BAY K8644 neither enhanced nor decreased response latency time when given alone (data not shown). When BAY K8644 (0.1 mg/kg i.p.) was administrated in ethanol-pretreated animals it significantly reduced ethanol (2 g/kg i.g.)-induced analgesia (Fig. 5).
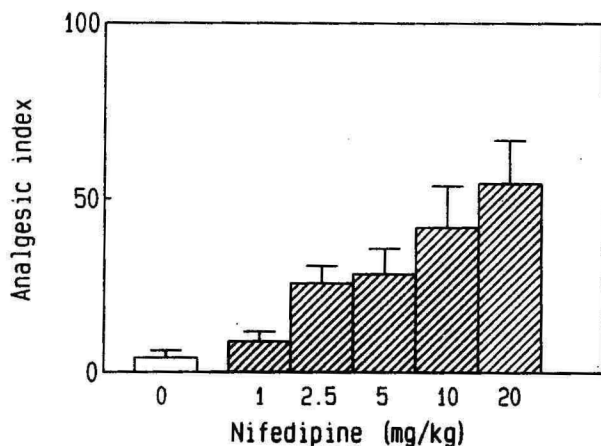
Fig. 1. Effect of nifedipine on hot plate response latency in rats. Each column represents mean ± SEM of 10 animals per group. Nifedipine was administered i.p. 60 min prior to test.
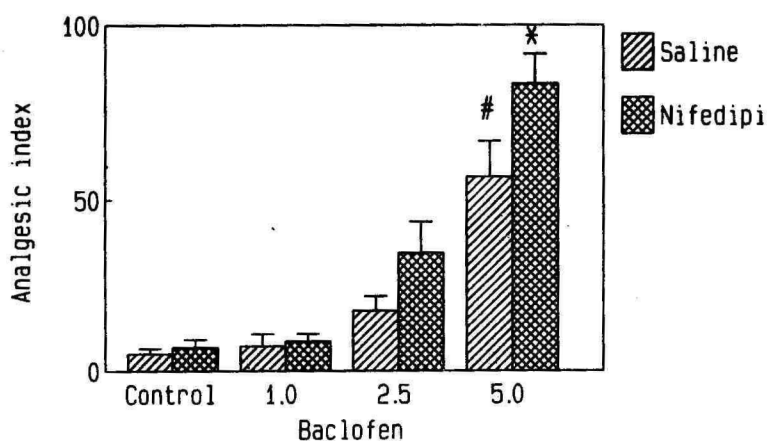
Fig. 2. Effect of nifedipine (1 mg/kg) on baclofen-induced analgesia. Each column represents mean ± SEM of 10 animals per group. Nifedipine and baclofen were administered 60 and 30 min prior to test, respectively.

\# - $P < 0.05$ as compared to control saline group,

* - $P < 0.05$ as compared to baclofen-treated group (multiple range test).

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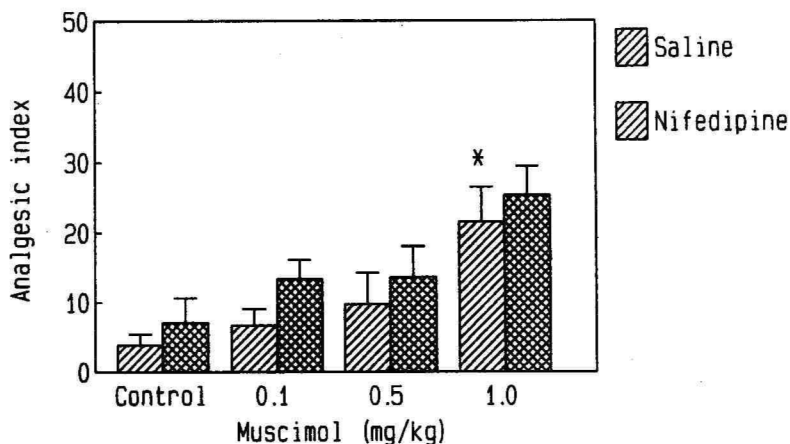
Fig. 3. Effect of nifedipine (1 mg/kg) on muscimol-induced analgesia. Each column represents mean ± SEM of 10 animals per group. Nifedipine and muscimol were administered 60 and 30 min prior to test, respectively.

* - $P < 0.05$ as compared to control saline group (multiple range test).
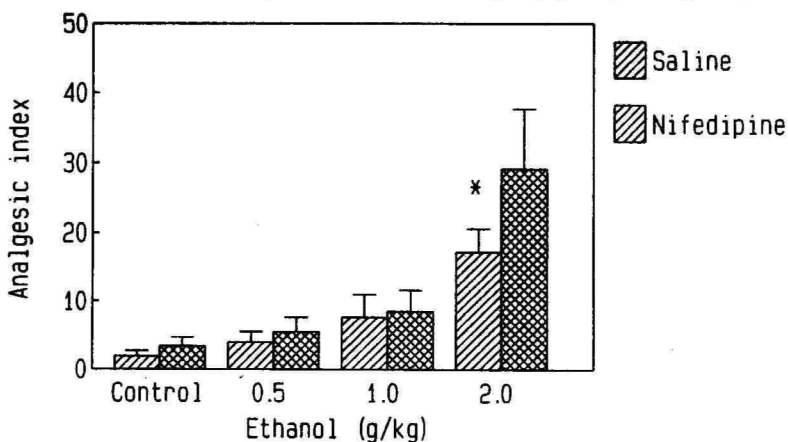
Fig. 4. Effect of nifedipine (1 mg/kg) on ethanol-induced analgesia. Each column represents mean ± SEM of 10 animals per group. Nifedipine and ethanol were administered 60 and 30 min prior to test.
* - $P < 0.05$ as compared to control saline group (multiple range test).

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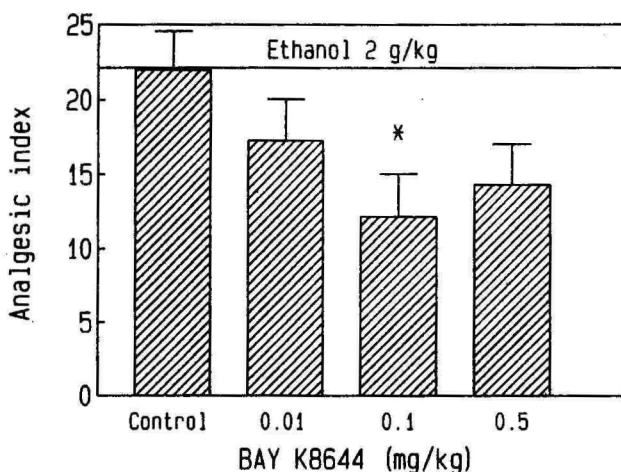
Fig. 5. Effect of BAY K8644 on ethanol (2 g/kg)-induced analgesia. Each column represents mean ± SEM of 10 animals per group. Ethanol and BAY K8644 were administered 30 and 15 min prior to test, respectively.

* - P < 0.05 as compared to control group (multiple range test).

Discussion

A number of evidence suggest that intracellular calcium plays an important role in the regulation of nociception and also in the analgetic action of different drugs such as opiates [20], baclofen [12] and ethanol [7].

In the present study, we found that DHP calcium channel antagonist nifedipine in a wide range of doses (2.5-20 mg/kg) exerts an antinociceptive effect in hot plate test. The observed analgesic effect of nifedipine is in agreement with previous studies, where it was found that calcium channel antagonists induce antinociceptive actions in mice when tested on the writhing induced by i.p. administration of acetic acid [6]. This effect of nifedipine is probably not linked to its vasodilatatory effect since a potent non-calcium-blocking vasodilating drug, hydralazine does not produce analgesia in the hot plate test [2].

A subanalgesic dose of nifedipine (1 mg/kg) when administered concurrently with $GABA_B$ receptor agonist baclofen resulted in a potentiation of baclofen-induced analgesia. As previously reported, baclofen acting at $GABA_B$ receptor either directly or indirectly affects calcium conductance [21]. On the other hand, co-administration of the same dose of nifedipine with $GABA_A$ receptor agonist muscimol did not produce any enhancement of antinociceptive effect of the latter. Muscimol has been proposed to increase chloride channel but not calcium channel conductance [13], therefore it could be speculated that the ability of nifedipine to enhance only baclofen- but not muscimol-induced analgesia as well as an analgesic effect exerted by higher doses of nifedipine alone may be related to its potency to inhibit calcium current via the voltage-dependent calcium channels. However, it should be noted that while dihydropyridine binding sites in the CNS may indeed represent functional Ca^{2+} channels [18], inhibition of calcium conductances following the activation of $GABA_B$ receptors may be a result of enhanced efflux of potassium with no direct action on calcium channels, as it has been shown in patch-clamp studies [10]. Nevertheless, there is yet another possibility for dihydropyridine binding site and $GABA_B$ receptor interaction. Namely, since the L-type calcium channel, which is the target for DHP calcium channel antagonists, is functional only when phosphorylated by protein kinase A [3] it would be natural to expect the L-type calcium channel to be modified indirectly by the $GABA_B$ receptor whose activation inhibits adenylate cyclase.

After pretreatment with nifedipine (1 mg/kg), there was a slight enhancement of analgesic effect of ethanol (2 g/kg) which, however failed to meet the significance criterion. The dihydropyridine calcium channel agonist BAY K8644 ethanol-induced analgesia. This "antiethanol" effect of BAY K8644 taken together with the minimal enhancement of ethanol-induced analgesia by nifedipine are in line with previous findings [8, 15] who showed that some behavioral effects of acute ethanol were augmented by calcium channel antagonists and reduced by calcium channel agonist BAY K8644.

In conclusion, although the exact nature of the interaction between GABA receptor agonists and calcium channel-affecting drugs of the dihydropyridine class remains to be elucidated , results obtained in the present study suggest that $GABA_B$ receptors and dihydropyridine binding sites could be functionally linked in the regulation of nociception possibly via voltage-sensitive calcium channels.

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CHANGES IN SENSITIVITY OF DOPAMINE AUTORECEPTORS AFTER SINGLE AND CHRONICMORPHINE ADMINISTRATION TO RATS

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Abstract

Yawning behaviour induced by quinpirole (0.05 mg/kg SC) in rats was used in order to study the state of dopamine (DA) autoreceptors after a single and chronic morphine administration. Acute morphine treatment (3.0 mg/kg SC) was given 15 and 90 minutes before assessment of yawning. Acute 15 minutes morphine pretreatment significantly inhibited yawning. On the contrary, acute 90 minutes pretreatment and chronic morphine administration significantly enhanced yawning. DA D1 receptor antagonist SCH23390 (0.01 mg/kg IP) given in combination with acute 90 minutes pretreatment, significantly increased the rate of yawning but failed to alter it in other experiments. DA D1 agonist SKF38390 in the dose 1.25 mg/kg had no significant influence on quinpirole induced yawning. The data obtained allow to conclude the state of supersensitivity of DA autoreceptors following single morphine administration (90 minutes pretreatment) and chronic morphine treatment.

Introduction

The biochemical and pharmacological evidence indicates the presence of two families of dopamine (DA) receptors, designated as D1-like and D2-like (12). The D1/D2 classification scheme is based primarily on the findings that D1 receptors are those which are linked positively to adenylate cyclase system whereas D2 receptors are not linked or inhibit this enzyme (5). The D2-like family contains D2, D3 and D4 subtypes (12). In addition to subdividing DA receptors on the pharmacological profile, one can also distinguish such receptors on the basis of their anatomical location. At least three DA receptors have been identified in this manner: postsynaptic receptors in apposition to DA nerve terminals, nerve terminal

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autoreceptors which modulate the synthesis and/or release of DA, and somatodendritic DA autoreceptors which regulate impulse flow in DA neurons by controlling K^+ conductance (7). DA auotoreceptors exhibit the pharmacological characteristics of DA D2-like receptors (16). Low doses of DA D2 receptor agonist preferentially affect DA autoreceptors and their activation is thought to induce sedation, sleep and yawning that reflects the inhibition of dopaminergic transmission (3,15,18).

It has been suggested that acute or chronic administration of morphine to rats could induce supersensitivity of postsynaptic DA receptors (10, but see also 6). As for DA autoreceptors, previous findings suggest rather a diminished DA autoreceptor control caused by morphine (2,3,16). Ahtee et al. (1989) demonstrated that withdrawal from chronic morphine administration decreased a striatal and limbic DA turnover, the challenge dose however, could increase it in withdrawn rats as well as in control group (3).

The aim of the present study was to investigate the state of DA autoreceptors after single and chronic morphine administration. As a behavioural model of activation of DA autoreceptors in rats yawning behaviour was used (but see also 9 and 11). Series of experiments (see table 1) was done in order to study the effect of acute and chronic morphine treatment (morphine alone and in combination with either DA D1 selective agonist SKF38393 or DA D1 selective antagonist SCH23390) on yawning behaviour induced by DA D2/D3 agonist quinpirole (LY171555)(13). Taking into consideration biphasic effect of morphine on locomotion in which DA receptors may have an essential role (9) yawning was assessed at various times (15 and 90 minutes) after acute morphine treatment.

Materials and methods

Animals: the experiments were carried out on male albino laboratory rats (Rappolovo Farm St.Petersburg) weighing 210-280g. The animals were kept in groups of 8-10 per cage and were given food and water ad libitum.

Drugs: Morphine Hydrochloride was dissolved in saline and injected SC (0.2 ml/100g) in the neck region (doses were calculated as a base). Quinpirole Hydrochloride (Ly171555) was dissolved in saline and injected 0.05 mg/kg SC (neck region). SCH23390 [R-(+)-8-chloro-2,3,4,5-tetrahydro-3-N-methyl-5-phenyl-1 H-3-benzodiazepine-7-ol hemimaleate; gift from Schering Corp., Bloomfield, USA] - was dissolved in a small amount of

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glacial acid and diluted with saline and injected 0.01 mg/kg IP 15 minutes before quinpirole injection. SKF38393 (2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1 H-3-benzazepine HCl; Smith, Kline and French Lab, Philadelphia, USA) was dissolved in warm saline and injected 1.25 mg/kg IP 5 minutes before quinpirole.

Table .1. Experiments done in order to investigate the state of DA autoreceptors after acute and chronic morphine administration. Abbreviations: S-saline, M-morphine, SCH-SCH23390, SKF-SKF38393, Q-quinpirole.

Experiment	Morphine treatment	Groups
E1	single dose 15 min. before test	S-S-Q S-SCH-Q M-S-Q M-SCH-Q
E2	single dose 90 min. before test	S-S-Q S-SCH-Q M-S-Q M-SCH-Q
E3	single dose 90 min. before test	S-S-Q S-SKF-Q M-S-Q M-SKF-Q
E4	chronic treatment, challenge dose given 90 min. before test	S-S-Q S-SCH-Q M-S-Q M-SCH-Q

To assess yawning the rats were placed into individual plexiglass boxes of 15X15X15cm 20 minutes before morphine injection. The number of yawns was counted during 30 minutes after quinpirole injection with a hand held counter.

Acute morphine administration experiments were arranged in randomized blocks: 32 male rats for each experiment were randomly divided into 4 groups (Table 1; n=8 per group) and during 4 days the tests were carried out (i.e. 2 animals from each group a day). When administered acutely morphine was given 3.0 mg/kg SC. All tests were carried out between 2-5 p.m.

Chronic morphine treatment: 32 male rats were randomly divided into 4 groups (Table 1; n=8 per group). Two groups received chronic morphine treatment and the remain of animals were administered chronically saline (0.2 ml/100g). Morphine and saline were given twice a day (8-9 a.m. and 4-5 p.m.) for 8 days, morphine in increasing daily doses: 20-30 mg/kg SC. The tests were carried out on the 9-th day between 2-5 p.m. (i.e. about 21 hours after the last injection). 90 minutes prior to assessment of yawning 3.0 mg/kg morphine was given to rats from morphine groups.

Statistical analysis: Experimental data organized in randomized block design were subjected to multifactor ANOVA. The experiment days (with 3 d.f.) were used as blocks. Two-Way ANOVA for chronic treatment was used. Post hoc multiple comparison was done using Tukey HSD test.

Results

Acute morphine administration: In E1 ANOVA revealed significant effect of 15 minutes morphine pretreatment [$F_{(1,25)}=28.4$, $p<0.0001$] and nonsignificant effects of SCH23390 and morphine X SCH23390 interaction. Further multiple comparison showed significantly lower number of yawns both in groups M-S-Q and M-SCH-Q than in group S-S-Q (fig.1). In group S-SCH-Q the number of yawns was slightly lower than in group S-S-Q.

On the contrary, 90 minutes morphine pretreatment significantly enhanced yawning both in E2 and in E3. By ANOVA was established significant effects of morphine: [E2: $F_{(1,25)}=20.9$, $p<0.001$; E3: $F_{(1,25)}=6.38$, $p<0.05$]. In E2 ANOVA revealed also significance of SCH23390 [$F_{(1,25)}=4.9$, $p<0.05$] and significant morphine x SCH23390 interaction [$F_{(1,25}=8.4$, $p<0.01$]. Further multiple comparison revealed significantly higher number of yawns in group M-S-Q versus other groups (fig.2). The number of yawns was slightly lower in group S-SCH-Q than in S-S-Q. In E3 ANOVA yielded nonsignificant effect of SKF38393. When compared with S-S-Q in group S-SKF-Q yawning was slightly enhanced (fig. 3).

Chronic morphine treatment (fig. 4) significantly enhanced yawning behaviour, Two-Way ANOVA revealed significance of morphine [$F_{(1,26)}=6.3$, $p<0.05$], nonsignificant effects of SCH23390 and morphine x SCH23390 interaction.

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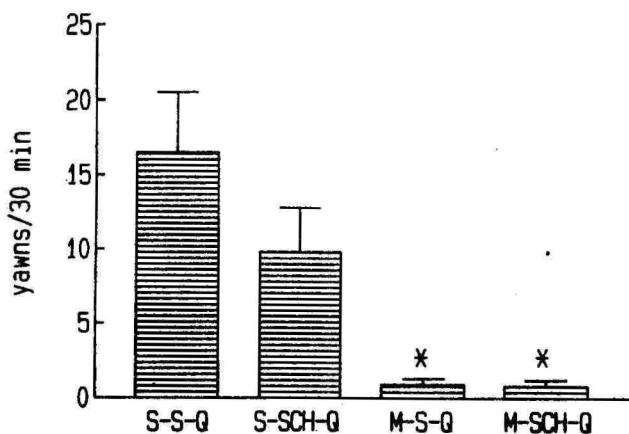
Fig. 1. Effect of 15 min. morphine pretreatment (3.0 mg/kg SC) and SCH23390 (0.01 mg/kg IP) on yawning induced by quinpirole (0.05 mg/kg SC). Each bar represents the mean ±SEM.
Abbreviations:
S – saline, M – morphine, SCH – SCH23390, Q – quinpirole.
*p<0.05 vs. group S-S-Q (Tukey HSD test).
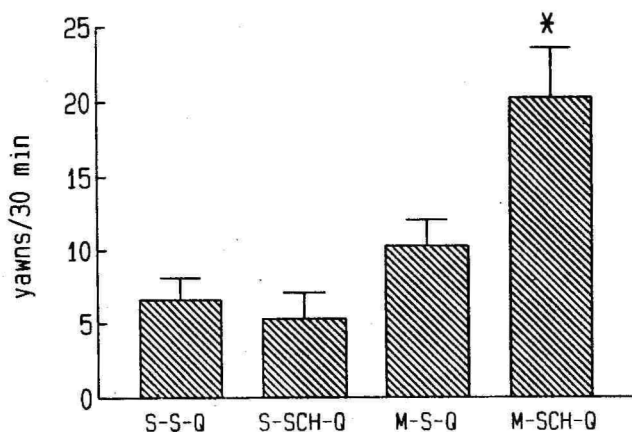
Fig. 2. Effect of 90 min. morphine pretreatment (3.0 mg/kg SC) and SCH23390 (0.01 mg/kg IP) on yawning induced by quinpirole (0.05 mg/kg SC). Each bar represents the mean ±SEM.
Abbreviations:
S – saline, M – morphine, SCH – SCH23390, Q – quinpirole.
*p<0.05 vs. other groups (Tukey HSD test).
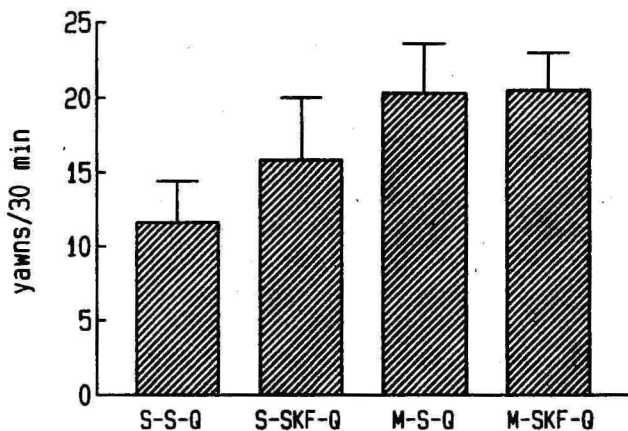
Fig. 3. Effect of 90 min. morphine pretreatment (3.0 mg/kg SC) and SKF38393 (1.25 mg/kg IP) on yawning induced by quinpirole (0.05 mg/kg SC). Each bar represents the mean ±SEM.
Abbreviations:
S - saline, M - morphine, SKF - SKF38393, Q - quinpirole.

Fig. 4. Effect of chronic morphine treatment and single administration of SCH23390 (0.01 mg/kg IP) on yawning induced by quinpirole (0.05 mg/kg SC). Each bar represents the mean ±SEM.
Abbreviations:
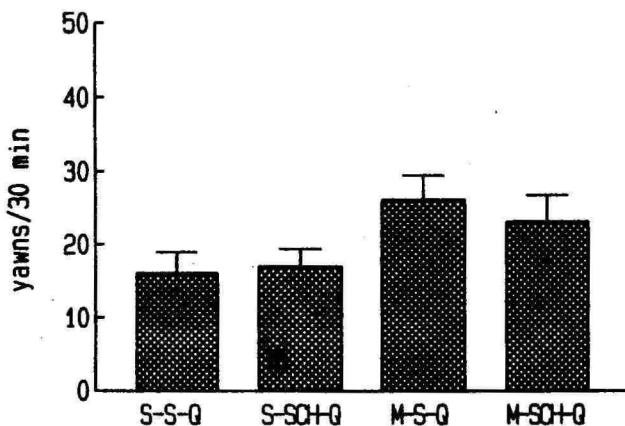S - saline, M - morphine, SCH - SCH23390, Q - quinpirole.

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Discussion

Acute 90 minutes morphine pretreatment significantly potentiated yawning induced by quinpirole. These results are interesting because previous findings suggest rather a subsensitive state of DA autoreceptors following morphine administration (1,2,16). Thus morphine has been shown to stimulate the firing of A10 DA cells leading to enhanced release of DA within the nucleus accumbens. Besides the subsensitivity of DA autoreceptors morphine has been shown to have direct inhibitory effects on the non-DA neurons in ventral tegmental area (VTA) that leads to inactivation of inhibitory GABA-ergic neurons within the VTA (4,8). In the present study stimulating effect on yawning of 90 minutes morphine pretreatment could be observed in two experiments (E2 and E3) thus, taking into consideration the autoreceptor origin of yawning a supersensitivity state of DA autoreceptors may be concluded.

A single dose of morphine given 15 minutes before test however inhibited yawning. One may explain it with concomitant increase of postsynaptic DA D1 receptors that exert inhibitory effect over DA autoreceptors mediated behaviours (18) but the opposite effects of acute 15 and 90 minutes pretreatment remain however, unclear. It has been shown the biphasic effect of morphine on locomotion: an initial hypomotility followed by hypermotility in which DA D1 receptors have an essential role (9). Hence, one should have presumed an enhanced influence of DA D1 receptors 90 minutes after morphine administration versus 15 minutes pretreatment and so results of the present study were an opposite kind to what could be expected.

DA D1 receptor antagonist SCH23390 when given in combination with 90 minutes morphine pretreatment significantly enhanced yawning (E2). These data are consistent with earlier studies that demonstrated the opposing role of D1 and D2 receptors in the regulation of yawning behaviour induced by low doses (0.025-0.15 mg/kg) of apomorphine (18). Interestingly in other experiments of the present study (E1 and E4) SCH23390 failed to alter yawning or even seemed to slightly decrease it, and just the opposite trend (slight increase of yawning) could be observed as the effect of DA D1 agonist SKF38393 in E3 [Morelli et al. (1986) demonstrated that SCH23390 given 0.05 mg/kg could antagonize yawning induced by apomorphine (11) and Spina et al. (1989) showed potentiating effect of SKF38393 on quinpirole induced yawning (14). Proceeding from those data the investigators concluded postsynaptic origin of this behavioural syndrome].

Chronic morphine treatment enhanced yawning induced by quinpirole. Although it has been shown that repeated opioid treatment did not cause tolerance to the opioid-induced increase of brain (except hypothalamus) DA turnover (2), it is very likely that in the present study the challenge dose of morphine (3.0 mg/kg) was too low in this respect. Then the data obtained are consistent with the earlier findings about decreased synthesis and release of striatal and limbic DA during morphine withdrawal.

Taken together, since yawning syndrome of rats is generally thought to be mediated via activation of presynaptic DA receptors (3,15,18), our results suggest that a single dose of morphine and withdrawal from chronic treatment could induce a supersensitivity state of DA autoreceptors.

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CHOLECYSTOKININ AND ANXIETY:
A BRIEF REVIEW

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Abstract

Central cholecystokinin (CCK)-ergic neurotransmission has been
implicated in the genesis of negative emotions. Most animal studies on the
neurochemical background of CCK-induced anxiety have, up to date,
exploited exploratory activity paradigms. Some recent studies, however,
have demonstrated that other paradigms of anxiety are also sensitive to
CCK receptor agonists and antagonists. The interaction of CCK with
GABAergic inhibitory neurotransmission, mediated probably through
CCK-B receptors, could be the neurochemical substrate for anxious
behavioural pattern. However, the CCK-A and CCK-B receptor-mediated
interactions of this neuropeptide with mesencephalic dopaminergic regula-
tion of motivation for locomotor activity have the potential to interfere
with the behavioural outcome from routine exploratory activity tests.
Systemic treatment with CCK receptor antagonists is likely to influence
both GABA- and dopamine-linked CCK-ergic neurotransmission, and
therefore their effects in exploratory activity tests should be interpreted
with caution.

Considerable amount of evidence is now available to suggest that
cholecystokinin (CCK) can act as a neurotransmitter in the central ner-
vous system [8], the octapeptide fragment (CCK-8)
being the predominant form of this peptide in the brain [51]. Neuronal
CCK has been proposed to play a significant role in feeding [44], pain
perception [3], memory [35] and sedation [62]. CCK exerts its effects
through interaction with specific receptors, that are currently divided into
two subtypes: CCK-A receptors in viscera and in a limited number of
brain regions, and CCK-B receptors that are widely distributed in the
brain [18,43]. Whereas the initial discrimination between "peripheral and
central" CCK receptors was based on the selectivity of unsulfated CCK-8
and shorter CCK fragments for "central" or CCK-B receptors, selective

nonpeptide antagonists for both receptor suntypes have been developed during the last years [21,34] which enable analyses of the contribution of both receptor subtypes in the regulation of behaviour.

CCK hypothesis of anxiety

Recently, neuroscientists have been fascinated by the idea that CCK might be an endogenous anxiogenic [50]. Indeed, CCK-related peptides have been shown to elicit anxiogenic-like effects in rodents [12,13,14,19,30,31]. CCK-8, caerulein, pentagastrin and CCK-4 have been used in these studies. The stimulation of CCK receptors can induce conditioned taste and place aversion, facilitate defensive burying, and impair exploratory behaviour in rodents. Moreover, cholecystokinin tetrapeptide (CCK-4) was recently characterized as a panicogenic agent in humans, being active in healthy volunteers [15] but especially potent in panic disorder patients [6]. This effect of CCK-4 is also shared with pentagastrin (CCK-5) [1].

Although distinct experimental approaches to study anxiety in animals are possible, not all of them can be equally favoured in the case of CCK. As a matter of fact, CCK exerts potent and complex anoretic effects which discourage the use of methods that are based on food or water motivation. Most studies have been designed to characterize the influence of CCK receptor ligands upon neophobic behaviour in several exploratory activity tests. However, the shortcoming of these techniques is the difficulty in making a clear distinction between behavioural reflections of changes in anxiety state and in general locomotor activity [24]. The latter depends strongly on the motivation of the animal to move from one place to another. Therefore anxiety induction, that is supposed to shift the ratio between exploratory and fear drives, may decrease exploration (as expected) only if such a motivation exists but is not too vital. On the other hand, extreme fear may cause startle reactions and an increase in locomotion measures (especially if the test time is short to avoid the effect of habituation) that must naturally not be attributed to anxiolysis.

It has been recognized for several years that CCK-peptides can depress exploratory activity, but perhaps due to their well-characterized sedative action [62], this was not usually interpreted as an anxiogenic action (what it, in most studies, probably wasn't, but in few cases possibly was). Recently, the decrease in exploratory activity after treatment with CCK-peptides has been observed in the elevated plus-maze test [12,13,23,26,31,53,58], by now one of the most popular anxiety measures [49].

Table 1 summarizes our data on the influence of some CCK-peptides on rat exploratory activity in two tests. It is clear that CCK-B receptors mediate the anxiogenic action of CCK in this species, whereas the stimulation of CCK-A receptors might be responsible for the more gross changes in general activity. Among these peptides, there is a high positive correlation between the affinity for CCK-B receptor and the potency as an anxiogenic [58]. Accordingly, CCK receptor antagonists should increase exploratory activity. Consistently, devazepide and L 365,260 are reported to display such an anxiolytic profile in plus-maze test [50], and similar effects have been obtained with the drugs recently synthetized at Parke-Davis that represent a chemically distinct class of CCK-B receptor antagonists [34]. In our laboratory, we have not been able to observe any anxiolytic-like effect of devazepide or L 365,260 as a single pharmacological treatment in elevated plus-maze test, however, both of these drugs (as well as proglumide and lorglumide) blocked the anti-exploratory action of CCK-4 (Table 2). Comparison of minimal effective doses of the CCK receptor antagonists against CCK-4 leaves little doubt that their action involves CCK-B receptors [31].

Table 1. Minimal effective doses of CCK receptor agonists in two behavioural tests: comparison with GABA-negative drugs

Drug	Receptor subtype selectivity	Elevated plus-maze	Open field
Caerulein	A & B	100 ng/kg	5 μg/kg
Pentagastrin	B	500 ng/kg	> 100 μg/kg
CCK-4	B	25 μg/kg	> 200 μg/kg
DMCM		0.3 mg/kg	1 mg/kg
Pentylenetetrazole		15 mg/kg	25 mg/kg

Elevated plus-maze test was performed according to the conventional methods [22,44] with minor modifications [24]; open field was a simple arena 1x1 m divided into 16 equal quadrants [24]. Parameters registered: plus-maze test, a) number of crossed sectors in the open part of the maze and b) time spent in open arms; open field test, a) number of crossed sectors and b) number of rearings. Minimal effective doses were defined as the doses which, 15 min after subcutaneous administration, decreased significantly (Mann-Whitney test, $P<0.05$) at least one of the two measures taken in a test.

CCK interaction with GABA

As far as the anxiety-related actions of CCK receptor ligands assume direct interaction with central GABAergic neurotransmission, the CCK receptor subtype might well be the CCK-B receptor: evidence for GABA/CCK-8 interactions comes from the studies of those brain regions (cerebral cortex, hippocampus) [5,27,30,61] where CCK-A receptors are not known to exist but CCK-B sites are rather abundant. Interestingly, these are also the brain regions where colocalization of GABA and CCK-8 has been described, whereas all or nearly all CCK-immunopositive neurons in these areas also synthetize GABA [32,37,54]. In the cerebral cortex, the release of CCK appears to be under tonic GABAergic control: not only GABA inhibits CCK release, the picrotoxin treatment can augment it. In hippocampus, CCK elicits fast excitatory postsynaptic potentials that can be blocked completely by low doses of benzodiazepines. More recently, coexistence of GABA and CCK was detected in the amygdala, another limbic structure [40], which could be the site of anxiogenic-like promnestic action of CCK-8, an effect that can be attenuated by benzodiazepine treatment [19].

Table 2. CCK receptor antagonists and exploratory activity of rats in the elevated plus-maze

Drug	Receptor subtype selectitivty	Previous anxiogenic treatment		
		None	CCK-4 50 μg/kg	Pictrotoxin 0.5 mg/kg
Proglumide	A & B	–	1 mg/kg	10 mg/kg
Lorglumide	A	.–	0.1 mg/kg	–
Devazepide	A	–	1 mg/kg	–
L 365,260	B	–	10 μg/kg	–
LY 288613	B	–	not tested	not tested

CCK receptor antagonists were given 5 min (proglumide) or 15 min (all others) before challenge with anxiogenics. Minimal active doses are given in the table - indicates the lack of effect. For further comments see the footnotes for Table 1.

In one study rats displaying anxious or non-anxious type of exploratory behaviour in plus-maze when tested in an undrugged state were separated into respective subgroups and radioligand binding to CCK-8 and benzodiazepine receptors was compared between these subgroups as well as to home-cage controls [26]. The results revealed that in anxious and non-anxious animals, a brief episode of forced exploration induced changes in receptor binding in these brain regions where CCK-8/GABA coexistence is observed (frontal cortex, hippocampus). These changes were different in anxious and non-anxious subgroups, further suggesting that CCK-B receptors are involved in anxiety-related alterations of exploratory behaviour. Moreover, pharmacological anxiogenic manipulations at GABA receptor complex (challenge with GABA-negative drugs at anxiogenic doses and diazepam withdrawal) also affect CCK-B receptor binding [26,27].

Elevated plus-maze test is not a simple anxiety test

However, during the past few years, besides the GABA hypothesis of anxiety, several other neurochemical mechanisms, most prominently serotoninergic ones, have been shown to be of major importance. Accordingly, it has been suggested that different aspects of anxiety deserve more attention and, perhaps, distinct animal models [22]. A very clear warning not to overemphasize the value of the studies on exploratory activity in the plus-maze test for understanding the brain mechanisms of anxiety comes from the evidence that azaspirodecandione anxiolytics (e.g. buspirone) show anxiogenic, if any, effect in this paradigm. Furthermore, according to the conventional characterization of the test [49], anxiety is expected to influence only few meassures in plus-maze, namely the percentages of open arm visits and time spent in open arms from total number of arm visits and total duration of the test, respectively. However, earlier [24] as well as recent [46] studies have shown that decrease in the total number of arm entries should not always be considered as a sign of a non-specific depression of activity. Indeed, for an aroused rat, the central platform of the plus-maze may frequently be a too dangerous place to enter. In open-field tests, there rats do not necessarily display any decrease in activity and there is no correlation between spontaneous activity of rats in elevated plus-maze and open field (our unpublished observations). The importance of central platform behaviour in mice was recently underlined and it was demonstrated that the platform was more aversive than the areas protected with walls [38]. Moreover, at certain doses diazepam may enhance the total number of entries [24], probably reducing the aversiveness central area.

The version of plus-maze most extensively used currently [24], after its minor modification and complex validation as a measure of anxiety [49] has been modified several times, e.g. by dividing the open arms into sectors to be counted as an index of open arm exploration [7,26]. It is notable that two groups of researchers in preclinical psychopharmacology independently have tried a similarly distinct approach from the established method. The conventional plus-maze test presumes that the open area is most fear-provoking when all the animal's four legs are out of closed arms as well as central platform. However, if the animal is really afraid of the open area, it will not enter the open arms placing all its four legs on it making the percentage calculations invalid (the number of entries into closed arms may vary in a great extent). Such a rat should not appear to be sedated and can approach the central platform several times [45]. The ratio of open arms entries and approaches to the central platform might therefore be more relevant index of anxiety in plus-maze, decreasing the problem of obtaining false negatives with anxiogenic drugs taken as sedatives. It even seems to fit best with the very first presentation of the idea of such an anxiety test [42], as the novel stimulation evokes "both the fear drive and the exploratory drive, thus generating an approach-avoidance conflict".

Focus on motivation: CCK and dopamine

The interest of neuroscientific community into neuronal CCK increased abruptly when CCK was demonstrated to colocalize with dopamine in a subpopulation of mesencephalic nerve cells, which innervate mesolimbic and cortical regions [29]. Studies on the interaction of CCK and dopamine in these neural pathways have been proceeding continuously. It is therefore regrettable that research directed at the role of CCK in anxiety is, despite taking advantage of exploration tests, missing the influence that CCK-peptides and CCK receptor antagonists might have on the dopaminergic mechanisms that are believed to possess a profound impact on motivations. Indeed, dependence-producing drugs increase DA release in nucleus accumbens (NAcc) [17] and increase locomotor activity, probably by activating mesolimbic DA system [55,57,60].

Considerable interest has been devoted to the role of NAcc in the CCK modulation of locomotor activity as this brain region is suggested to be the specific site for the action of CCK-peptides [36]. Administration of CCK-8 directly into NAcc may lead to either an increase [11] or decrease [10,20,36] in the locomotor activity of rats. CCK-8-induced decreases

in locomotion are elicited through CCK receptors in the posteromedian part of NAcc which probably are of CCK-A subtype [12,13,14]. The behavioural effect is dependent on the motivational state of the animal, as CCK-8 decreased activity only in rats that were not habituated to the environment [13]. Indeed, intra-NAcc CCK potentiates DA action, having no effect alone [9]. The behavioural effects of CCK-8 and CCK-4, the unselective and B-subtype selective agonists respectively, after administration into NAcc, are clearly opposite: in the studies where CCK-8 was described to decrease locomotor activity [36] or intracranial self-stimulation [16], CCK-4 increased both. The neurochemical mechanisms that cause such an opposite regulation of behaviour via distinct receptor subtypes are not precisely identified, but recently it was demonstrated that CCK has a dual action on potassium-stimulated dopamine release from NAcc, causing an increase in dopamine release via CCK-A receptors and decrease via CCK-B receptors [39]. The latter effect may be the basis for the anti-amphetamine effect of CCK-8 in NAcc [9], whereas potentiation of DA-induced hyperlocomotion, probably CCK-A-mediated action, implies modulation of postsynaptic DA receptors.

In one important study [19], the ability of CCK-8 to induce behavioural arousal was linked amygdala, whereas its neuroleptic-like effects were associated with nucleus accumbens. The importance of NAcc in the integration of limbic output to locomotor behaviour is generally recognized [41]. An intriguing question is how the NAcc-controlled locomotion is linked to the effects of anxiety on exploration measures. Present knowledge supports the hypothesis, according to which CCK has multiple roles in the regulation of exploratory behaviour: 1) in the brain regions, where it colocalizes with GABA, it participates in the genesis of anxiety; 2) in NAcc, it modulates the DA-dependent motivational control over ongoing behaviour. These two CCKergic systems may be interrelated, for example, through excitatory input to NAcc from amygdala and hippocampus that has been shown to decrease exploratory activity [41]. Interestingly, the basolateral nucleus of amygdala, one of the sources of this input, has been suggested to be a site of action for benzodiazepine anxiolytics [52] and is one of the regions of GABA/CCK coexistence [40].

From Table 2, it is apparent that only proglumide (the unselective CCK receptor antagonist) reliably blocked the anxiogenic-like effect of picrotoxin on exploratory activity. The explanation for this can be derived from the above hypothesis, suggesting that the blockade of both CCK-A and CCK-B receptors that participate in GABA/CCK and DA/CCK interactions might be necessary. This is in accordance with the idea that picrotoxin can reduce exploratory drive at the same time as enhancing fear

[24]. The primary importance of CCK-B receptor blockade is simple to derive from most of the studies conducted to date; however, this leaves open the possibility of an action of CCK-8 On CCK-A receptors in posteromedian NAcc, shown to decrease exploration in novel environment [13].

Other neurotransmitters involved?

It is certainly inappropriate to restrict attention only to the interaction of neuronal CCK-ergic activity with GABA and dopamine. For example, nucleus accumbens receives afferents from 5-HT-ergic dorsal raphe cells [56], and most recently an excitatory influence of CCK-8 on 5-HT neurones in the rat dorsal raphe has been demonstrated [4]. The receptors mediating this effect of CCK-8 appear to be of CCK-A subtype and could, in parallel to peripheral CCK-A receptors [48], serve as a target for the sedative action of CCK-peptides. We have recently been able to demonstrate, that the anti-exploratory effect of caerulein can be attenuated by ondansetron, a representative 5-HT-3 receptor antagonist [59]. Since this effect was observable only in a limited dose range, interference with general locomotor effects should also be considered here. As a matter of fact, lower doses of ondansetron had diminishing influence on locomotion, possibly masking the anti-anxiety action.

Another alley yet to be explored is the interaction between CCK and noradrenaline. If NA-ergic nerve terminals are damaged by treatment with DSP-4, the neurotoxin that selectively damages the projections from the locus coeruleus, surprisingly few changes can be observed in gross behaviour. What has consistently been shown to change, however, is the exploratory behaviour and adaptation to novelty. Since the upregulation of CCK receptor binding in the rat frontal cortex has been correlated with deficits in plus-maze behaviour, it was of interest to investigate the possibility that the degeneration of the locus coeruleus input leads to alterations in the CCK receptor binding abilities. This has indeed turned out to be the case [25]. DSP-4 treatment caused, in a dose-dependent manner, significant increase in the density of CCK-8 binding sites in those brain regions, that receive their major NA-ergic input from the locus coeruleus (frontal cortex, hippocampus), but not in those, where there are few locus coeruleus projections (hypothalamus, corpus striatum). The time-course of the development of changes in CCK-8 binding paralleled with some lag the development of changes in noradrenaline uptake, whereas desipramine pretreatment abolished both. These findings demonstrated that denervation

of noradrenergic input from the locus coeruleus induces certain alterations in the CCK-ergic neurotransmission, similar to those seen in rats with deficits in response to novel stimuli. CCK could therefore mediate the neophobic responses observed in animals after lesions of noradrenergic innervation of the forebrain.

CCK and suicide

There is significant overlap between panic disorder and self-destructive behaviour, and also other anxiety disorders and suicide [47]. Among the dimensions of personally, related to suicide, susceptibility to fear/anxiety appears, but according to a psychobiological model of self-destructive behaviour, suicide is unlikely to occur even in a highly vulnerable individual unless the person finds himself in a situation which he/she conceives as desperate [2]. Suicidal behaviour could, thus, be categorized as an ultimate desadaptation with environmental changes. Animal research had led us earlier to suggest that high density of CCK receptors in cortical regions might be a basic biological correlate of deficits in adaptation with environment [26]. Thus, one might wonder whether brain CCK-ergic transmission is affected in suicidal persons. This question was addressed in a study carried out on post-mortem brain samples from 19 suicide victims and 23 well matched control cases [28]. In the frontal cortex, significantly higher apparent number of CCK receptors and affinity constants were found in the series of suicide victims. These differences between suicides and controls were present in similar proportions when the suicide cases with depressive syndrome or violent or non-violent means of self-killing were compared to matched controls. However, when the samples were split into subgroups consisting of persons either below or over the age of 60 years, significant differences in the CCK receptor characteristics in the frontal cortex were observed only between younger suicides and controls. Furthermore, the younger suicide victims had a higher density of CCK receptors in the cingulate cortex, whereas in older suicides the value was lower as compared to age-matched controls. It should be noted that the cortical CCK receptor binding basically does not change neither in aged rats nor in aged humans [28,29]. No difference in benzodiazepine receptor binding was found between control and suicide groups. The results of this investigation suggest that CCK-ergic neurotransmission is linked to self-destructive behaviour, probabaly through its impact in anxiety and adaptational deficits. Since severe anxiety, at variance with depression, is uncommon in the elderly, and

panic disorders is very rare among the old people, CCK might well have distinctr roles in the neurochemistry of self-destructive behaviour in different age groups.

CCK agonists as anxiogenics of choise for further research?

CCK-B receptor agonists offer one clear advantage as test anxiogenics. As far as conventional anxiogenic drugs also possess (at higher doses) a potential for convulsant action, increasing the dosage has usually resulted in further decreases in gerneral activity, possibly due to effects rather unspecific to exploratory drive. CCK-B agonists, however, appear to represent a group of drugs that may induce anxiety and perhaps increase neophobia, but do not decrease the motivation to move and do not prevent the animal from changing location. CCK-4 has also turned out to be a safe and efficient anxiogenic for the humans, especially potent in case of clinically detectable emotional disturbances.

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SOCIAL ISOLATION MODULATES THE SENSITIVITY OF CHOLECYSTOKININ RECEPTORS IN THE RAT BRAIN

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Abstract

The role of CCK receptors in the development of anxiety caused by social isolation of rats was studied using the elevated plus-maze and receptor binding techniques. The isolation of male Wistar rats significantly reduced their exploratory activity in the elevated plus-maze compared to that in the rats kept in groups of four. Caerulein (0.1-5 μg/kg s.c.), an agonist of CCK receptors, dose-dependently decreased the exploratory activity in the plus-maze test of the rats housed in groups, but not that of single rats. Caerulein even tended to increase the behavioural activity of isolated rats.

In parallel to the behavioural changes, isolation of the rats increased the number of [^{3}H]pCCK-8 binding sites in the frontal cortex, but not in the other forebrain structures (the mesolimbic area, striatum and hippocampus). Isolation did not affect the density of benzodiazepine receptors in the frontal cortex.

In conclusion, the isolation of rats for 7 days produced anxiogenic-like effect on the behaviour of rats and increased the number of CCK-8 receptors in the frontal cortex without affecting benzodiazepine receptors.

Key words: Isolation - Caerulein - CCK receptors - Benzodiazepine receptors - Rat

Introduction

Compared to the rats kept in social groups, isolated rats have various behavioural disturbances, like increased locomotor activity in an open field test and deficits in both learning and spatial memory tasks (Morgan et al. 1975; Sahakian et al. 1977; Morinan and Parker 1985). In addition, the exploratory activity of these animals is markedly decreased in the elevated plus-maze (Wright et al. 1990). In the white-black box preference test, animals spend most of their time in the dark compartment (Morinan et al. 1992).

Significant alterations in dopaminergic and serotonergic transmission in various brain structures of isolated rats have been shown. Isolated rats show greater increases in the frontal cortex dopamine metabolism in response to foot-shock (Blanc et al. 1980). Dopamine release stimulated by amphetamine is also augmented (Jones et al. 1988) compared to the animals kept in groups. Enhanced efflux of cortical serotonin to local application of KCl or systemic treatment with fenfluramine has been reported in isolated rats (Crespi et al. 1992). In contrast, the density of benzodiazepine receptors remains unaffected by social isolation (Morinan et al. 1992).

Recently the role of cholecystokinin (CCK) has been established in the regulation of anxiety-related states in laboratory animals and man (Ravard and Dourish 1990; Harro and Vasar 1991b). The administration of CCK-4, an agonist at the central subtype of CCK (CCK_B) receptors, suppressed the exploratory activity of rats in the elevated plus-maze test (Harro and Vasar 1991a). CCK-4 also induced panicogenic effect in the man (Bradwejn et al. 1990). The selective CCK_B antagonist L-365,260 has been demonstrated to reverse the anti-exploratory (Harro and Vasar 1991a) and panicogenic actions of CCK-4 (Bradwejn et al. 1992). Taking into account these observations an attempt was made to study the role of CCK receptors in the development of suppression of exploratory behaviour after the social isolation of rats. Since CCK and GABA have obvious functional and morphological interactions (Somogyi et al. 1984; Bradwejn and De Montigny 1984) the properties of the benzodiazepine receptors were also studied.

Materials and Methods

Animals. Male Wistar rats, weighing 180-200 g at the beginning of experiment, were caged individually or in social groups of four.

Behavioural testing of both groups, as well as collecting of brain samples for radioligand binding studies, took place 7 days following the different housing.

Elevated plus-maze. The method initially described by Handley and Mithani (1984) for measuring of exploratory activity in rats was used with some modifications (Harro et al. 1990c). Briefly, the plus-maze consists of two opposite open arms (50x10 cm) without side walls and two enclosed arms of the same size with 40 cm high side walls and end wall. The arms extended from a central platform (10 x 10 cm) and were angled at 90^6 to each other, making a shape of a plus sign. To determine the exploratory activity in the open part of plus-maze the pair of open arms (together with the central platform area) was divided by lines into 7 equal squares. The maze was elevated to the height of 50 cm. During a 5 min observation session the following measures were taken by observer: 1) latency period of the first open part entry (the entry of the animal with two forelimbs into the central platform), 2) number of line crossings in the open part (open arms + central platform area), 3) total time spent in the open part of plus-maze, 4) number of closed and open arm entries, and 5) ratio between open and closed arm entries. At the very beginning of the experiment an animal was placed into the centre of the plus-maze facing to the closed arm. Rats did clearly prefer the enclosed arms. An arm entry was counted only when all four limbs of the rat were within a given arm. Each animal was used in only one experiment. The precaution was taken to control the possible daily fluctuations in the exploratory activity of animals. Therefore, the experiments were always performed in random- ized order, i.e., the rats in groups were always used in parallel with singly housed rats. Caerulein (0.1-5 µg/kg s.c., Bachem, Bubendorf, Switzerland) was injected 15 min before the beginning of the plus-maze experiment.

Radioligand binding experiments. Rats (8 isolates and 8 animals kept in social groups) were decapitated and the brains were quickly dissect- ed. Brain tissue was homogenized in 20 volumes of ice-cold Tris-HCl (pH 7.4 at 4°C) using a Potter-S glass-teflon homogenizer (1000 rpm, 12 passes). The membranes were washed twice in the same buffer by centrifu- gation and resuspension. For CCK-8 binding experiments the pellets were homogenized in HEPES buffer (10 mM HEPES; 130 mM NaCl; 5 mM KCl; 1 mM EDTA; pH 6.5 adjusted with 5 N NaOH) containing bovine serum albumin (0.5 mg/ml). CCK-8 receptor labelling was carried out in the presence of 0.05-2.4 nM tritiated ligand, [propionyl-^{3}H]propionylated-CCK-8-sulphated ([^{3}H]pCCK-8, specific activity 79 Ci/mmole, Amersham Radiochemicals) at room temperature in a total incubation volume of 0.5 ml. Caerulein (100 nM) was added to determine nonspecific binding. For
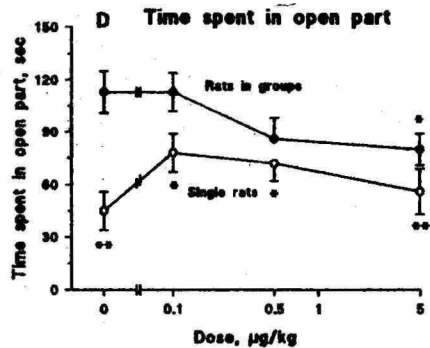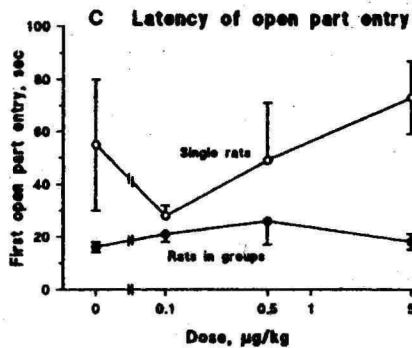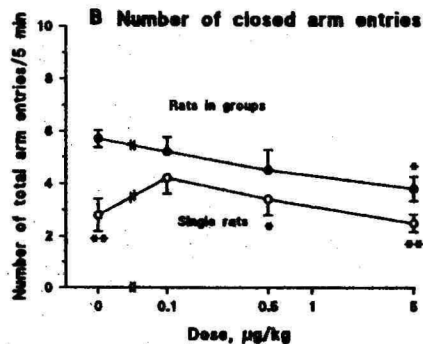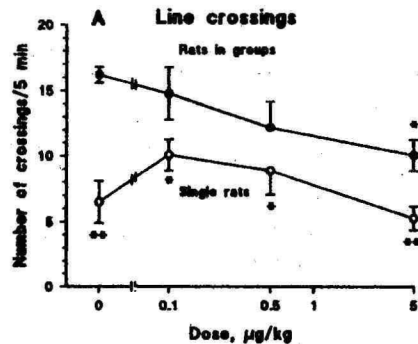
benzodiazepine receptor labelling the pellets were homogenized in Tris-HCl buffer and the membrane preparations incubated in the presence of 0.25-16 nM N-methyl-[^{3}H]-flunitrazepam (specific activity 84 Ci/mmole, Amersham Radiochemicals) at 4°C in a total volume of 0.5 ml in the presence or absence of unlabelled diazepam (10 μM) to determine nonspecific binding. Incubation was terminated after 120 min ([^{3}H]pCCK-8 binding) or 60 min ([^{3}H]-flunitrazepam binding) by rapid filtration over Whatman GF/B filters using a Brandel Cell Harvester (M-24S). The filters were washed with 10 ml cold incubation buffer, dried and assayed for radioactivity by liquid scintillation spectrometry. The protein content was measured according to a modification of the Lowry procedure (Markwell et al. 1978). Saturation curves were analyzed using non-linear least squares regression (Leatherbarrow 1987).
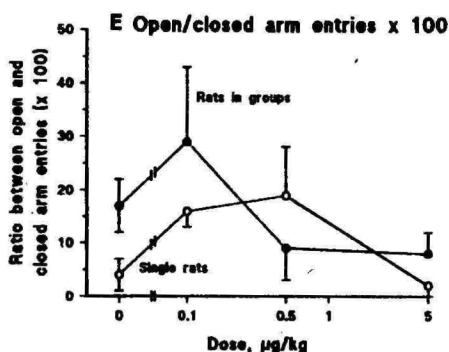
Statistics. Two-way analysis of variance followed by Duncan's multiple range test was used for evaluation of the behavioural data. Student's t-test for paired observations was used to determine statistical significance of the results obtained from the radioligand binding experiments.

Results

The social isolation of rats for 7 days reduced nearly all the behavioural parameters registered in an elevated plus-maze (Figure 1, two-way ANOVA, $F_{1,88}$=27.02, P<0.01 line crossings; $F_{1,88}$=21.24, P<0.01 time spent in open part; $F_{1,88}$=15.84, P<0.01 enclosed arm entries; $F_{1,88}$=7.50, P<0.05 open arm entries; all compared to the respective values of the rats kept in groups of four). Latency of first open part entry was increased in isolated animals (two-way ANOVA, $F_{1,88}$=10.28, P< 0.01). The ratio between open and enclosed arm entries was not changed significantly, probably owing to the limited number of open arm entries made by control animals: 0.9 ± 0.3 entries during the observation time.

The administration of caerulein produced statistically significant reduction of line crossings (Figure 1, two-way ANOVA $F_{3,88}$=3.54, P<0.05) and enclosed arm entries (two-way ANOVA $F_{3,88}$=2.83, P<0.05). The comparison of the action of caerulein upon the exploratory behaviour of single housed and group-housed rats revealed the difference only in the case of the time spent in open part (two-way ANOVA $F_{3,88}$=3.01, P<0.05). Accordingly, in single housed rats caerulein at lower doses (0.1 - 0.5 μg/kg) tended to increase time spent in exploration of open parts compared to that in rats kept in groups.

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A Line crossings
Rats in groups
Single rats
Number of crossings/5 min
Dose, µg/kg

B Number of closed arm entries
Rats in groups
Single rats
Number of total arm entries/5 min
Dose, µg/kg

C Latency of open part entry
Single rats
Rats in groups
First open part entry, sec
Dose, µg/kg

D Time spent in open part
Rats in groups
Single rats
Time spent in open part, sec
Dose, µg/kg

Figure 1. Effect of social isolation upon antiexploratory action of caerulein in rats.

Part A. Number of line crossings. Saline treated rats kept in groups made 16.1 ± 0.6 line crossings. .

Part B. Number of closed arm entries. Saline treated rats kept in groups did 5.7 ± 0.55 closed arm entries.

Part C. Latency of first open part entry. This value of saline treated group-housed rats was 16 ± 2 s.

Part D. Time spent in open part. Saline treated group-housed animals spent 114 ± 11 s in open part.

Part E. Ratio between open and closed arm entries. This value of saline treated group-housed rats was 16 ± 5.

Caerulein was injected 15 min before the beginning of the experiment. $^{+}$ - $P < 0.05$ (Duncan's multiple range test after significant two-way ANOVA, comparedto saline treated group-housed animals).
* - $P < 0.05$; ** - $P < 0.01$ (compared to the respective values of group-housed rats).

[^{3}H]pCCK-8 binding sites in the frontal cortex (B_{max}) were increased in the isolated rats compared to rats in groups (P<0.05). No such a difference was seen in the other forebrain regions (mesolimbic area, striatum and hippocampus) (Table 1).

On the contrary, the isolation of rats did not affect the parameters of [^{3}H]-flunitrazepam binding in the rat frontal cortex. In rats kept in groups of four the dissociation constant of [^{3}H]-flunitrazepam binding was 1.65 ± 0.22 nM and the apparent number of binding sites was 1096 ± 88 fmol/mg protein, whereas in isolates these values were 1.95 ± 0.29 nM and 1262 ± 94 fmol/mg protein, respectively.

Table 1. Effect of rats' social isolation upon [^{3}H]pCCK-8 binding in the rat forebrain

Brain structure	Rats in groups		Single rats	
	K_d	B_{max}	K_d	B_{max}
Cerebral cortex	0.60±0.05	11.7±0.79	0.71±0.03	15.0±1.28
Mesolimbic area	0.61±0.04	12.5±0.73	0.60±0.02	12.9±0.89
Striatum	0.47±0.03	12.4±0.73	0.39±0.03	13.1±0.62
Hippocampus	0.73±0.10	6.5±0.64	0.71±0.11	5.8±0.62

The number of rats in both groups was 8.
K_d, dissociation constant (nM);
B_{max}, apparent number of binding sites (fmol/mg protein).
Statistics: P < 0.05 as compared to grouped rats

Discussion

The isolation of rats immediately post-weaning has been shown to suppress the exploratory behaviour of animals in an elevated plus-maze (Wright et al. 1990). However, in our present study already the 7-day isolation of sexually mature rats markedly inhibited the exploratory activity of animals in the plus-maze test. Since it is well-known that various anxiogenic drugs inhibit the exploratory behaviour of rodents (Harro et al. 1988, 1989), we are tempting to conclude that the isolation of rats induced an anxiety-like state in these animals. In parallel binding studies, the number of [^{3}H]pCCK-8 binding sites was elevated in the frontal cortex of isolated rats compared to the rats kept in groups. This

result could support the view that CCK-ergic mechanisms contribute to the development of anxious behaviour in the rat (Harro and Vasar 1991b).

Since dopaminergic and serotonergic systems are also affected by social isolation (Blanc et al. 1980; Jones et al. 1988; Crespi et al. 1992), there may be important interactions between various neurotransmitter systems. This interplay could be related to the dense morphofunctional interaction of CCK-ergic and the major monoaminergic systems in the brain. The colocalization of dopamine with CCK in the mesencephalic neurons, innervating mainly the limbic and cortical regions, is widely accepted (Hökfelt et al. 1980). On the other hand, the intra-cerebroventricular administration of CCK-4, a selective CCK_B agonist, affected the metabolism of serotonin in several brain regions (Itoh et al. 1988) and increases the density of serotonin$_2$-receptors in the cerebral cortex (Agnati et al. 1983).

CCK has also a considerable functional interaction with GABA-ergic mechanisms in the cerebral cortex and hippocampus (Somogyi et al. 1984). However, no eventual changes in the parameters of benzodiazepine receptors have been found (Morinan et al. 1992; the present study). Moreover, a benzodiazepine agonist diazepam is not able to block the anxiogenic-like effect of caerulein, an unselective agonist at CCK_A/CCK_B receptors, in the plus-maze test (Harro et al. 1990a; our unpublished data). This seems to indicate that GABA-ergic system is not involved in the isolation-induced anxiety in the rat.

It is noteworthy that the most, if not all, of CCK-8 binding sites in the frontal cortex belong to the CCK_B receptor subtype (Hill et al. 1987). Various anxiogenic manipulations with rats have been demonstrated to cause the up-regulation of $[^3H]pCCK$-8 binding sites in the frontal cortex. Indeed, the systemic administration of the anxiogenic compound FG 7142, an inverse agonist at benzodiazepine receptors, and the withdrawal of long-term benzodiazepine treatment increased the number of CCK_B receptors in the frontal cortex (Harro et al. 1990b, c). This finding was also supported by the plus-maze selection study, where the animals having the low exploratory activity by nature have significantly higher density of CCK_B receptors in the frontal cortex compared to their 'non-anxious' counterparts (Harro et al. 1990c). Now we found that also a social isolation of rats increased the number of CCK_B receptors exclusively in the frontal cortex. These findings may have some significance for human anxiety, since CCK receptor density in the frontal cortex of suicide victims is also higher than in matched controls (Harro et al. 1992).

The anti-exploratory effect of caerulein, the unselective agonist at CCK_A/CCK_B receptors, was not visible in the plus-maze test in the ani-

mals isolated for 7 days. The lack of the effect of caerulein in the isolated rats could be explained by the above finding that the animals with the 'anxiogenic-like' behaviour had a higher density of CCK_B receptors compared to behaviourally more active rats. It may be that in these 'anxious' rats, an endogenous CCK alone is able to suppress nearly maximally the exploratory behaviour. Similar unresponsiveness of animals to the anti-exploratory effect of caerulein has been established in mice after withdrawal of long-term diazepam treatment, when the baseline anxiety of animals was high (Harro et al. 1990a).

A hypothesis of the importance of endogenous CCK and particularly the increased number of the cortical CCK_B receptors in anxiety is further supported by a recent study where CI-988, a selective antagonist at CCK_B receptors, blocked the behavioural signs after benzodiazepine withdrawal (Hughes et al. 1990). Since much lower doses of caerulein are needed to block the exploratory activity of rats after peripheral administration (Harro et al. 1990a; Vasar et al. 1992) if compared to the central application of CCK agonist (Singh et al. 1990), it is doubtful that the peripherally administered caerulein really could interact with the CCK_B receptors in the cerebral cortex. Rather, CCK_B receptors in the nucleus of solitary tract could be the targets for the anxiogenic-like effect of CCK agonists (Branchereau et al. 1992), assuming that there is a relation between the density of CCK_B receptors in the cerebral cortex and the sensitivity of CCK_B receptors in the brainstem structures.

In conclusion, the isolation of sexually mature rats for 7 days induces in these animals the anxiety-like state. This behavioural change is associated with the increased number of CCK_B receptors in the frontal cortex of rats without any effect on benzodiazepine receptors.

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MODIFIED GATING KINETICS OF CARDIAC SODIUM CHANNELS AS A POSSIBLE CAUSE OF ISCHEMIA-INDUCED DEPOLARIZATION

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Abstract

The ionic current mechanisms underlying ischemic arrhythmias have been the subject of great interest and investigation. We studied cardiac myocytes treated with the ischemic metabolite lysophosphatidylcholine (LPC) using both the cell-attached and excised inside-out patch clamp technique at 22 oC. In addition to previously recognized LPC-induced non-inactivating Na channels we have found that larger concentrations of LPC and/or more prolonged incubation time induces Na channels openings at potentials negative to usual Na channel threshold, that could be described as complete removal of inactivation and shift in voltage-dependence of activation of Na channel. Both kinds of modified Na channels may play a role in development of cardiac cells depolarization during ischemia. At the other hand, they could be the important targets for antiarrhythmic drugs.

Introduction

Ischemia-induced depolarization (ID) of myocardial cells known as one of the major phenomena responsible for cardiac arrhythmias (12). The loss in resting potential results in inactivation of sodium channels, slowing of excitation conduction, reentry circles formation and abnormal automaticity development.

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At least two different mechanisms of depolarization thought to be involved in ID development. First one is failure of regenerative repolarization and the appearance of new steady level of membrane potential (Vm) close to action potential (AP) plateau voltage level. Such phenomenon has been found in ischemic Purkinje fibers (22), in Purkinje fibers damaged by the isolational procedure (1) or treated with ischemic metabolites (2). Second mechanism of ID is well-known gradual ID associated with extracellular K accumulation recognized in both in vivo and in vitro ischemia (see for review Reference 12). The electrophysiological mechanisms of both kinds of ID are not completely known.

Lysophosphatidylcholine (LPC), toxic metabolite of sarcolemmal phosphatidylcholine, has long been suspected to be one of the mediators of acute ischemic changes in heart (7). LPC induces electrophysiological alterations (including both types of ID) and arrhythmias which closely parallel those observed in early ischemia (7). We had previously shown that LPC decreases probability of Na channel opening and induces a slowing of Na channels inactivation (2, 4, 22). We report here that larger concentrations of LPC and/or more prolonged incubation time induces Na channels openings at potentials negative to usual Na channel threshold, that could be described as complete removal of inactivation and shift in voltage-dependence of activation of Na channel. Some of these data have been presented in abstract form (21). We will try also to discuss plausible role of LPC-induced alterations of Na channel gating in ID development.

Methods

Channel recordings were made from enzymatically dispersed ventricular cells of adult rat heart isolated by a method similar to that described elsewhere (20). Single - channel currents were recorded using patch-clamp technique (9) in cell attached and inside-out configurations. LPC (Serva, 9-13 uM) was applied to the inner side of inside-out patches. Some experiments were made using the cells preincubated in LPC-containing solution. Cell suspension was exposed to LPC (20 uM) in storage solution for 1 - 3 h before experiments were done. Storage solution contained (mM): NaCl, 135; KCl, 5.4; MgCl2, 1.2; CaCl2, 0.3; HEPES buffer, 5; glucose, 10; pH 7.2. Solutions used in cell attached experiments: bath solution had the same composition as the storage solution, except the NaCl was replaced with KCl with the purpose to depolarize cells; pipette solution solution had a similar composition as the storage one but KCl was replaced by CsCl. In inside-out experiments bath solution contained (mM): CsF, 100;

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KCl, 34; NaCl, 3.6; EGTA, 10; HEPES-KOH buffer 10; pH 7.2. The pipette solution had a similar composition as a pipette solution for cell attached experiments. Experiments were performed at room temperature 21 - 23oC.

Results

Application of depolarizing pulse (Fig. 1A) from holding potential (Vh) of -120 mV to -20 mV (close to action potential plateau) results in simultaneous opening of Na channels at first few milliseconds. After that Na channels have been opened as well as non-opened channels become inactivated and don't open despite the maintained depolarization. Nevertheless, an extremely little number of Na channels openings could be observed at later times due 1) delayed first openings of some channels; 2) reopenings, i.e. second openings of channels once have been opened (16); 3) burst-like openings of the channels operating in kinetic mode with modified gating (18).

Application of LPC (9-13 uM) to inner side of inside-out patch markedly increases the probability of appearance of Na channels demonstrated burst-like gating (Fig. 1B). Frequency of bursts and burst length progressively increased with time and at 30-60 min of LPC application the bursts lasted up to end of 300- ms depolarizing pulse. Thus at that time clamping of membrane back to Vh rapidly cease any Na channel activity. Holding of membrane at more positive Vh of -60 mV resulted in complete elimination of both control and burst-like activity. We have concluded that short-term (up to 1h) LPC exposure provoke Na channels to operate in mode with slower entrance in inactivation state.

More prolonged LPC exposure (1-3 hours, exposed was cell suspension) resulted in appearance of completely non-inactivating sodium channels seems to operating in wide range of membrane potentials (-180 mV - +60 mV). Such channels activity was recognized in both cell attached (Fig. 2) and inside-out (not shown) patches from LPC-preincubated cells. In contrast to Ca-activating cation-unspecific channels described earlier (5), resting Na channels functioned in presence of 10 mmol/l EGTA from inside and have potential-dependent gating kinetics (Fig.2). Reversal potential for these channels interpolated from single-channel voltagecurrent relationship was close to Na equilibrium potential predicted by Nernst equation.

Discussion

Because low doses of tetrodotoxin (TTX) shorten the plateau of AP and induce the slight but statistically significant hyperpolarization in cardiac Purkinje fibers without affecting the upstroke velocity, Coraboeuf with coworkers (6) suggesting that there are at least two sodium currents that are different from the one underlying AP upstroke. Further step in understanding the nature of sodium current taking part in AP plateau maintenance was made by Patlack and Ortiz (18) demonstrated that cardiac sodium channels in extremely small percent of sweeps could operate in burst-like mode. Respecting high density of sodium channels (especially in Purkinje fibers, Reference 8), despite low probability of burst-like behavior, the current through these channels might be substantial and contributive in AP plateau maintenance.

Marked increase in probability of burst-like channel activity found after short-term LPC application might induce imbalance between inward and outward ionic currents determining AP plateau formation resulting in progressive AP prolongation and, finally, in second stable voltage level development (2). Such loss of regenerative repolarization thought to be one of the prominent mechanisms of ischemic arrhythmogenesis (22). Preferential blockade of noninactivating Na channels by TTX (13) and lidocaine (10) could explain found property of these agents to normalize repolarization (11) and, possibly, beneficial antiarrhythmic properties of latter in early ischemia.

Recently obtained data provide the basis for critical review of the proposed mechanisms of gradual ID development. In particular, it has been shown that extracellular K accumulation is rather the result of enhanced passive K efflux than a decrease in cells K uptake by Na-K pump (15, 19). Na-K pump remains its functional activity, at least during the early ischemia (first 10 - 15 min). The hypothesis that decreased gK at RP level (for example, due LPC-induced decrease of inward rectifier potassium channels conductance, Reference 14) could induce gradual ID seems to be incorrect, because this actually opposes the well described increased net K efflux observed in ischemia. The alternative hypothesis that ID is a following of increased K conductance (due to ATP-sensitive potassium channels partial activation, see Reference 17) also seems to be controversial. The increased gK would cause the membrane potential to more accurately approximate the K equilibrium potential and at least initially would cause hyperpolarization. In addition, the driving force for K efflux would be reduced thus diminishing K accumulation.

A

2 pA
50 ms

B

2 pA
50 ms
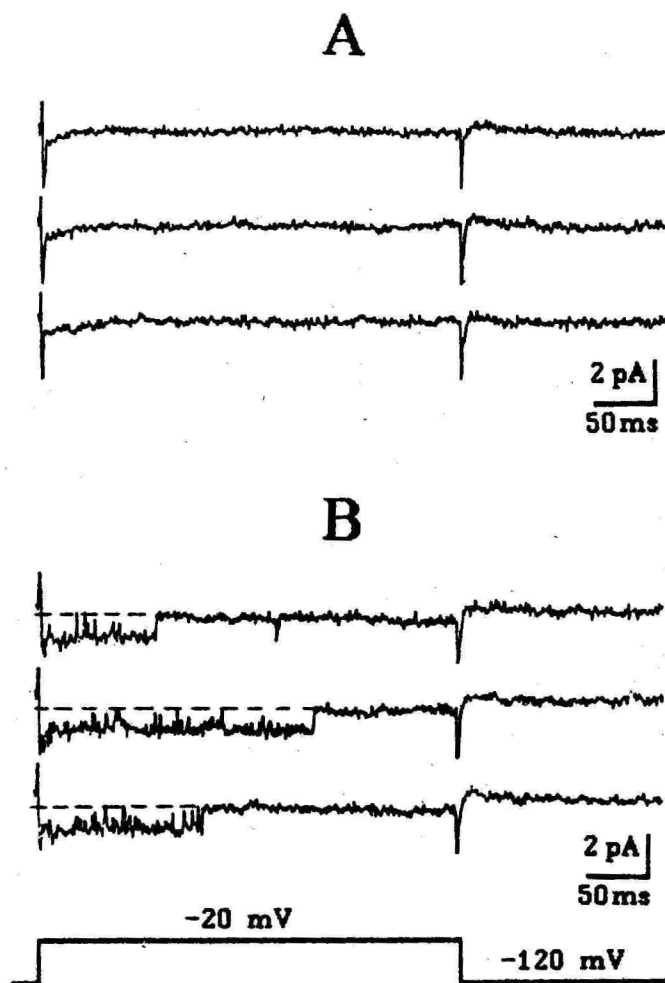
-20 mV

-120 mV

Fig.1. Successive records of Na channels currents from inside-out membrane patch in control (A) and after 15 minutes of LPC (10 uM) application from inside (B). Holding potential was -120 mV and 100 mV, 300 ms voltage clamp pulses were applied with the frequency of 1 Hz. Leakage and capacitive currents were partially subtracted only by analog circuitry. Downward deflection of current just after voltage pulse onset corresponds to simultaneous openings of Na channels. Note that after LPC treatment (B) one of the Na channels demonstrate burst-like activity up to about 200 ms after pulse onset.
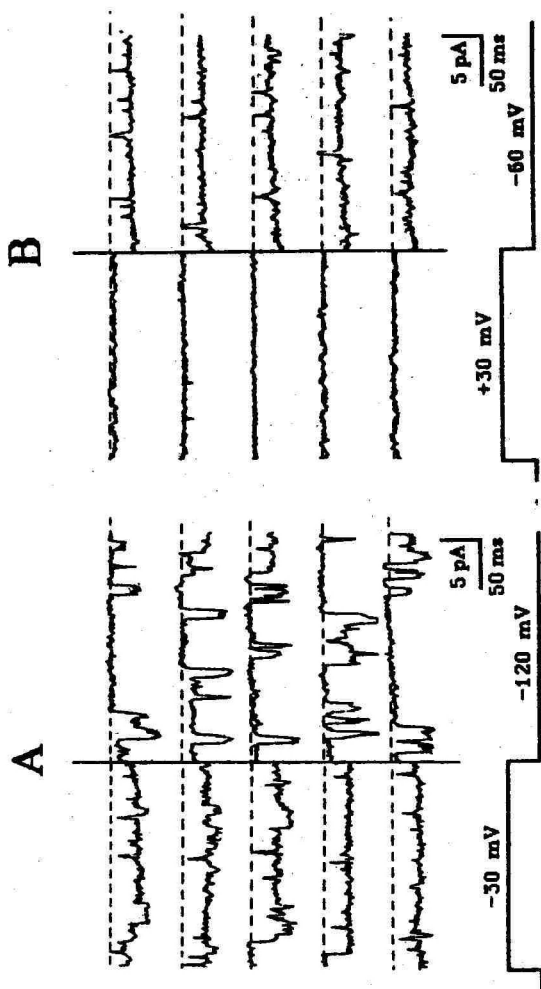
Fig.2. Na channels currents recorded in cell attached membrane patch from LPC-preincubated cardiomyocytes. LPC (20 uM) treatment lasted 1.5 h in storage solution (see Methods). Currents were elicited by 90 mV, 200 ms voltage steps from holding potentials of -120 mV (A) or -60 mV (B). Note that in both cases channels activity remained after membrane was clamped back to holding potential (in contrast to less prolonged LPC exposure, Fig. 1). Note also a clear voltage dependence of resting Na channels gating kinetics.

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This discrepancy might be overcame if initiation of additional small but permanent inward current close to RP voltage is postulated. Small depolarization inducing by this current could result in appropriate increase in K current. Substantially decreased blood flow and increased net K efflux could provide unbalance in transmembrane K fluxes and increase in extracellular K concentration leading to further depolarization.

The candidates to the role of such inward current are 1) cation-unspecific Ca-activated channels (5); 2) resting Na channels described here.

Defining the role of modified Na channels in ischemiainduced arrhythmogenesis may be useful for developing therapeutic strategies to deal with the leading cause of mortality from ischemic heart disease.

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PLASMA MEMBRANE RECEPTORS
AS TARGETS FOR CANCEROGENS
(minireview)

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This minireview highlights several facts which clearly indicate the crucial role of the plasma membrane and its components (especially receptors) in cancerogenesis.

The first viewpoint: **the plasma membrane, via its components, is intimately involved in the cell proliferation and cancerogenesis.**

Elucidation. It is widely accepted that several cancerogens interact with DNA to form its structural and functional changes. These alterations cause changes in gene products, which generation is followed by uncontrolled proliferation and cancer development [1, 9]. Besides, most current antitumor drugs (e.g. anthracyclines, platinum complexes, alkylating agents etc.) are directed also against DNA or/and nucleic acids biosynthesis. On the other hand, several cancerogens, including different oncogenes, cause some structural-functional alterations of the plasma membrane. It is noteworthy, that above-mentioned drugs cause these alterations already at therapeutic doses [13]. Consequently, the plasma membrane and its components role in precise mechanism of several cancerogenic and anticancerogenic agents is really more important than has been appreciated. For example, it has been shown an important role of plasma membrane receptors and associated signaling pathways in the molecular mechanism of chemical cancerogenesis [3, 11]. Moreover, there is shown also a significant correlation between the degree of receptor binding and cancerogenic activity among a series of cancerogens [4, 5]. Additionally, the cancer initiation and promotion involve modulation of several receptor and second messenger systems. Among of them growth factor receptors and corresponding signaling pathways, including receptors for EGF (epidermal growth factor), PDGF (platelet derived growth factor), insulin-like growth factor; bombesin etc., are clear targets for certain cancerogenic agents [7, 11].It is also accepted, that this receptor family (characterized by the

tyrosine kinase activity) is involved in cell proliferative response via several intracellular events: phosphorylation of intracellular proteins, activation of phospholipase C, stimulation of inositolphosphatides breakdown, activation of proteinkinase C, elevation of free cytosolic Ca^{2+} etc. It is noteworthy, that somehow or other all these events are associated with structural-functional alterations of the plasma membrane.

The second viewpoint: **in fact, only manifold and repeated character of the influence of cancerogens on the plasma membrane components underlay the development of cancer.**

Elucidation. It is conceivable, that repeated activation of growth factor receptors and/or associated pathways by the cancerogenic "agonists" (mitogens) leads by different molecular mechanisms to the activation of corresponding oncogenes and to the uncontrolled cell proliferation. It seems likely, that many cancerogens amplify proliferative response, caused by endogenous growth factor signals. For example, simultaneous exposition of pulmonary neuroendocrine (PNE) cells to different mitogens - chronic hypooxygenation (or hyperoxygenation) and nicotine (or nitrosamines) induces neuroendocrine lung tumors[10]. On the other hand, cancerogenic "agonist" binding with its "specific" receptor can affects several other receptors and associated pathways, especially those of muscarinic and adrenergic receptors as it is demonstrated in the case of methapyrilene [4]. It is important to consider that some cancerogens realize their modulatory effects only via intracellular messenger systems. Thus, phorbol esters family can activate proteinkinase C (component of many growth factor signaling cascade) and therefore amplify the physiological mitogenic response initiated at the receptor level [3]. At the same time some chemical cancerogens (e.g. nitrosamines) can mimic such physiological ligands as serotonin, catecholamines, acetylcholine and several peptide hormones and affect of cAMP-dependent protein kinase (I isozyme) which is actively proliferating enzyme in normal and cancerous tissues [8].

The third viewpoint: **the role of the lipids in cancerogenesis and in cancer therapy is more important than has been appreciated.**

Elucidation. Many of plasma membrane essential components (several ionic pumps, receptors etc.) are lipoproteins. Consequently, the lipid status of the plasma membrane regulates significantly the functionality of these systems [13 ,etc.]. Besides, several growth factors, including PDGF, EGF, bombesin and thrombin, stimulate a phoshatidyl inositol-4,5-biphosphate (PIP_2) specific phospholipase C. This is followed by generation of inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). It is noteworthy, that PIP_2, IP_3 and DAG are lipids. In fact, lipid (IP_3)

causes the release of Ca^{2+} in cell and lipid (DAG) activates protein kinase C and both these events are important mitogenic signals [1].

The platelet activating factor (PAF) is also lipid. Recently, by the cloning of the receptor cDNA from guineapig lung, the existence of a membrane receptor for PAF has been shown [6]. Evidently, this is the first lipidmediator receptor gene to be cloned and it belongs to the superfamily of G-protein-coupled membrane receptors [2, 15]. Synthetic analogues of PAF - ether lipids- are phospholipids analogues and they inhibit the crucial signal transduction enzymes: protein kinase C and phospholipase C. As these ether lipids directed against the plasma membrane and do not affect DNA [15], such kind phospholipids analogues are a new class of antitumor agents which action mechanism realizes via an interference with plasma membrane components functions.

Additionally, the precise action mechanism of the oncoproteins remains still unclear. However, some data allowed to suggest that the action of oncoprotein E5 is mediated via plasma membrane lipids or their products [12]. It is noteworthy, that lipids free radicals may play the role of certain second messengers in this pathway.

We really hope, that consideration of these viewpoints is valuable for researches both in the field of receptors theory and in clinical elaboration of new antitumor agents for cancer therapy.

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POSSIBLE MECHANISMS OF
GALANIN NEUROTROPIC ACTIVITY

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Galanin, a 29 amino acid long C-terminally amidated peptide, is widely distributed in central and peripheral nervous system. Binding studies with ^{125}-galanin have demonstrated the highest galanin receptor (GR) concentration in hippocampus and hypothalamus [9]. However, the precise action mechanism of galanin remains still unclear. The present study aims to characterize the GR-mediated membrane events (alterations of adenylatcyclase and Na-pump activities), triggered by this neuropeptide in different rat brain regions.

Materials and methods

For the adenylate activity determination male albino rats (weighing 200-250 g) were killed by decapitation. Their brains were removed rapidly. From the precooled brains were isolated three regions: frontal cortex, hypothalamus and hippocampus. Immediately after that the brain regions were homogenized in 4 volumes of ice-cold 10 mM Tris-HCl (pH 7,4), homogenates were diluted 1:15 with mentioned buffer, mixed on ice for 30 min and centrifuged for 6 min at 1600 g. Supernatants were discarded and pellets resuspended in ice-cold buffer PB, containing 30 mM Tris-HCl, 1,5 mM theophylline, 8,25 mM $MgCl_2$, 0.75 mM EGTA, 7,5 mM KCl, 100 mM NaCl (pH 7.4) to get a protein concentration 0,5-1.0 mg/ml. Adenylate cyclase activity was measured by determining the formed cAMP and expressed as pmoles cAMP /min per mg of protein. For the assay of basal activity membrane proteins (final concentration 0.03-0.04 mg/ml) incubated in the reaction buffer, containing above-mentioned PB, 0,1 mg/ml bacitracin, 0.05% human serum albumin, 0.01 mM GTP, 1,0 mM ATP and an ATP-regenerating system. Galanin (10^{-6}M) effect was assessed in the same incubation mixture. Assay of forskoline (10^{-5}M) stimulated adenylate cyclase was introduced for more exact determine of galanin effect. Enzyme reaction was initiated by the addition of ATP and terminated after a 15

min incubation at 30°C by adding 100 mM EDTA and boiling of samples for 3 min in a water bath. Protein precipitate was removed by centrifugation at 600g. Aliquots of supernatant were used for the cAMP measurements by [2]. Bovine adrenal glands were used as the source of high specific cAMP-binding protein.

Protein determination was performed according to [6] using bovine serum albumin as a standard.

Basal adenylate cyclase level in cortex, hypothalamus and hippocampus was respectively 86±3, 111±3 and 77±7 pmol cAMP/min per mg protein.

The enzyme preparations (membrane fragments) of the Na,K-ATPase from different rat brain areas were isolated as previously described [4]. The enzyme activity estimation, the treatment with phospholipase A_2 (PLA_2) and SDS were performed according to procedure described earlier [5]. The determination of the phospholipids (PL) and cholesterol (CH) were performed as described in literature [5].

Student's t-test for paired observations was used to determine statistical significance of experimental results.

Results

As shown in table 1 the basal adenylatcyclase activity of hypothalamus and hippocampus was poorly activated by galanin: 23±7% and 13±3% respectively. Although the enzyme from frontal cortex was also activated, this effect was not statistically significant.

Unlike to basal adenylate cyclase the forskolin-stimulated enzyme was significantly inhibited by this peptide (table 1). The inhibition was more remarkable in the case of hypothalamus (59%) and hippocampus (50 %). Small inhibition was also established in frontal cortex (11%).

In connection with mentioned above is noteworthy, that gala- nin inhibits the rat brain Na-pump . As is shown in table 2 this inhibition was incomplete. Besides, the inhibitory effects were different in various brain areas and there was a definite correlation between the content of PL and CH in various brain areas and the inhbition degree.

Table 1. Galanin (10^{-6}M) effect on the basal and forskolin-stimulated adenylate cyclase activities in rat brain frontal cortex, hypothalamus and hippocampus.

Brain region	Adenylate cyclase activity (% of basal value)			
	Basal	Basal + Galanin	Forskolin-stimulated	Forskolin-stimulated + Galanin
Frontal cortex	100±3	112±5	409±3*	363±3*
Hypothalamus	100±2	113±3*	408±3*	167±6*
Hippocampus	100±9	123±7*	397±2*	196±3*

The data are means ± S.E.M. from 4-8 experiments and expressed as percentage of the respective basal (control) values (100 %).
 * - statistically significant (P< 0.01) alterations of adenylate cyclase activity.

Table 2. The inhibitory effect (%) of the galanin (10^{-3} M) on the activity of the rat brain Na,K-ATPase and the content of PL (μmol P_i/mg protein) and CH (arbitrary units) in the enzyme preparations.

	Galanin*	+PLA$_2$	+SDS	PL	CH
Frontal cortex	40±8	8±2	5±2	1.04	11.8
Substantia nigra	44±7	3±2	-	1.17	13.0
Hippocampus	54±7	5±2	3±2	1.05	9.3
Striatum	37±6	-	-	0.92	7.6
Nucleus accumbens	19±3	-	-	0.69	7.0

 * all galanin's effects were statistically significant (P<0.05)

Discussion

The low activating effect of galanin on basal adenylate cyclase activity in hypothalamus and hippocampus may refer to a direct binding of the peptide with its receptors, coupled to the cAMP production system. In fact, it is shown that GR high concentration is namely in hypothalamus and hippocampus [6,9]. Additionally, galanin binding with other receptors of brain is evidently unlikely, because this peptide is not a member of neuropeptides family known nowadays [8]. However, some data show the existence of negative coupling-mechanism (via G_i) between GR and adenylate cyclase in several organs and tissues [1,6]. One explanation of this phenomenon is following: a tissue- and/or species-specific cofactor which is needed for inhibition) of adenylate cyclase by galanin in vivo, is absent in our experiments (disappears during membrane preparation or misses in the enzyme assay medium).

This study shows inhibitory effect of galanin on the forskolin-stimulated brain adenylate cyclase similar to that obtained in several other observations [1,6]. Evidently, such effect is due to the conformational changes of the oligomeric enzyme after the treatment by this cardioactive diterpene. Obviously, conformation state of adenylate cyclase, induced via forskolin binding (with the enzyme catalytic unit), supports the inclusion of G_i in the mechanism of GR adenylate cyclase interaction.

In many aspects, our observation suggests that cAMP production system as well as other functionally active membrane systems (ion-channels and ion-pumps) in plasma membrane are important transmembrane signaling pathways for galanin's effect in various brain regions. At the same time, the regulative action of bioactive ligands realizes evidently in situ via several mechanisms. It means that the regulative effects of such ligands may be mediated via plasma membrane lipids and their active products. For example, it is suggested recently, that in suitable lipid medium of the plasma membrane the exact, strong and effective binding of the ligands is guaranteed [3]. The lipoproteinous nature of the plasma membrane active systems mentioned above and incompleteness galanin's effects on them refer to possibility that the lipid-protein interactions may play a definite role in regulative mechanism of this peptide. Our such suggestion is supported also by following facts: 1) the inhibitory effect of galanin disappears practically after the treatment with delipidative agents - PLA_2 and SDS (table 2), 2) the lack of ATP protective effects against the influence of galanin or PGE_2(lipohilic modificator of brain Na-pump) on the brain Na,K-ATPase [5], 3) the lack of competition between the galanin and the regulative ions (sodium and potassium) in the case of brain

Na,K-ATPase [5], 4) a definite correlation between the effect of galanin and the Na,K-ATPase preparations lipid status (table 1 and [5]).

This study shows small regulative effects of galanin on brain basal adenylate cyclase and Na,K-ATPase activities, which is in agreement with the other investigations. Futher studies are required to clarify whether molecular mechanism of this neuropeptide is in situ coupled to other membraneous and/or intracellular transduction systems (e.g. lipids).

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FREQUENCY OF IMPAIRED HYDROXYLATION OF MEPHENYTOIN AND DEBRISOQUINE IN AN ESTONIAN POPULATION

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Abstract

Debrisoquine and S-mephenytoin hydroxylation polymorphisms were studied in 156 unrelated native Estonians. The hydroxylation phenotypes were assessed by coadministration of mephenytoin with debrisoquine or dextromethorphan. The frequency of the poor metaboliser phenotype of debrisoquine/dextromethorphan was 4.5 % (95 % confidence interval 1.2 - 7.8 %), and that of mephenytoin was 3.9 % (95 % confidence interval 0.9 - 6.9 %) among Estonians, which is very similar to that in other Caucasian populations.

Introduction

Interest in polymorphic drug metabolism has grown rapidly over the last 15 years. Since the description of the genetic polymorphisms of debrisoquine/sparteine [5] and S-mephenytoin [20], several important interindividual differences in drug responsiveness can be explained by polymorphic drug oxidation [5,20]. Pronounced interethnic differences between Caucasian and Oriental populations in the hydroxylation polymorphisms have been demonstrated [12].

It is considered that most European Caucasians have evolved from a Caucasoid descendant genetically distinct from the progenitors of Oriental populations [4]. Nevertheless, in this worldwide reconstruction of human evolution, some European populations (Basques, Finns, Hungarians and Estonians) seem to differ both genetically and linguistically from the others. Previous studies of polymorphic drug metabolism among Hungarians [17] and Finns [2,16] were limited to debrisoquine hydroxylation

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phenotyping and did not reveal significant differences from the average European population [1].

It has been shown that coadministration of debrisoquine and mephenytoin [13], or dextromethorphan and mephenytoin [8], does not interfere with the phenotyping test results. Also, the probe drugs debrisoquine and dextromethorphan [9,14] identify the same activity of the debrisoquine hydroxylase (cytochrome P4502D6). Therefore, a combination approach was used to study prevalence of hydroxylation polymorphisms in a previously uncharacterized population of Estonians.

Methods

A total of 156 unrelated Estonians (55 men and 101 women, age 20 to 31 years) were recruited from medical students of the Tartu University. The study group consisted of Caucasian Estonians of at least second generation living in Estonia. The subjects were interviewed and considered to be in good health, and they participated in the study after giving oral and written informed consent. Subjects had not experienced any recent ilness, and were not taking any medication, except some had taken antibiotics or acetylsalicylic acid.

After emptying his or her bladder at bedtime, each subject took a 10 mg tablet of debrisoquine (Declinax[R], Hoffman-LaRoche; 78 subjects) or 30 mg dextromethorphan in 15 ml syrup (Tussidyl[R], Tika; 78 subjects) together with a 100 mg tablet of racemic mephenytoin (Mesantoin[R], Sandoz; all subjects). Urine was collected for 8 h, the total volume recorded and 10 ml stored at -20° C until analyzed in Huddinge, Sweden.

The mephenytoin S/R ratio in urine was determined by chiral gas chromatography according to [18] and modified by us [13]. In urine samples in which R-mephenytoin, but not S-mephenytoin could be detected, the S/R ratio was defined as the lower level of detection (0.05). The mephenytoin poor metaboliser (PM) phenotype was confirmed by acidification procedure according to [19], and a subject was assigned a PM phenotype in case acidification did not change the urinary S/R ratio.

Debrisoquine (DEB) and 4-hydroxydebrisoquine (4HDEB) in urine were analysed by the gas chromatographic method by [11]. The metabolic ratio was calculated by dividing the molar concentrations of DEB and 4HDEB.

The concentrations of dextromethorphan (DXM) and dextrorphan (DO) in urine were determined by high performance liquid chromatography (HPLC) after hydrolysis of glucuronidated DO. Hydrolysis: 0.5 ml of

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urine was mixed with 100 μl of internal standard (640 μg disopyramid phosphate in 10 mM hydrochloric acid), 10 μl of ß-glucuronidase from Helix Pomatia type H-2 (Sigma), and 1.0 ml of 0.1 M sodium acetate buffer pH 5.0. The mixture was incubated in a shaking waterbath for 16 h. Extraction: the incubated sample was mixed with 2.0 ml of 0.5 M ammonium sulphate pH 9.3 (adjusted with ammonia), and extracted for 10 min with 5.0 ml of diisopropyl ether. Four ml of the organic phase was then extracted on a whirlmixer for 10 s with 200 μl of 50 mM phosphoric acid. HPLC : 20 μl of the phosphoric acid phase was injected onto a 10 cm reversed phase column with 4.6 mm inner diameter, containing 3 μm Spherisorb Phenyl (Phase Sep, Dessida, UK). The eluent was a 10 mM potassium dihydrogenphosphate buffer pH 3.0 (adjusted with phosphoric acid) containing 25 % of acetonitrile. The flow-rate was 1.5 ml/min. Ultraviolet detection was carried out at a wavelength of 210 nm. Retention times were 2.6 min for DO, 4.2 min for disopyramid (internal standard), and 6.5 min for DXM. Standard curves were obtained by analysis of urine spiked with up to 4.0 μM of DXM, and up to 80 μM of DO. Peak heights were measured. The standard curves were linear with correlation coefficients of >0.998 (six points). The lower limits of detection were about 0.05 μM for both DMX and DO. The metabolic ratio was calculated by dividing the molar concentrations of DXM and DO.

Two-tailed Students t-test was used for statistical analysis of group parameters. Conventional statistical procedures were applied to calculate standard deviatons and 95 % confidence intervals [7] for the prevalence of the two drug hydroxylation phenotypes.

Results

Six of the 156 investigated Estonians (3.9%, 95% confidence interval 0.9 - 6.9%) had a mephenytoin S/R ratio ranging 0.93 - 1.44, indicating that they were PM of S-mephenytoin (Table 1 & Fig. 1). Of these, 3 were available to be retested with mephenytoin, and the second night urine (24 - 32 h) was collected. The S/R ratios 0.94 - 1.03 in this fraction confirmed that they were PM. The 0-8 h urines from the other 3 potential PM were acidified without any change in S/R ratio confirming that they also were PM.
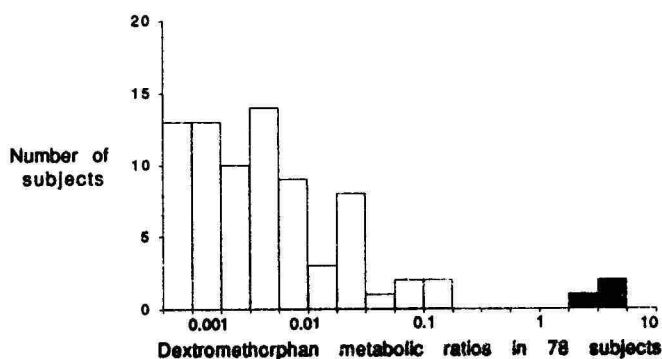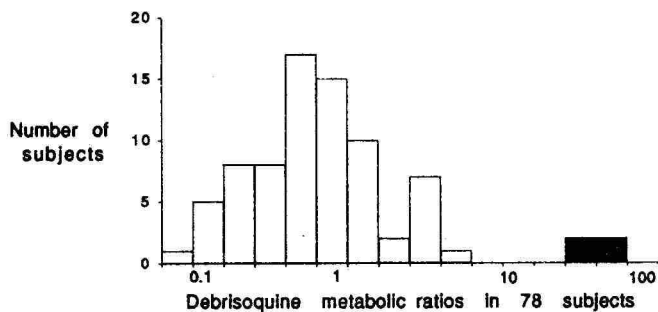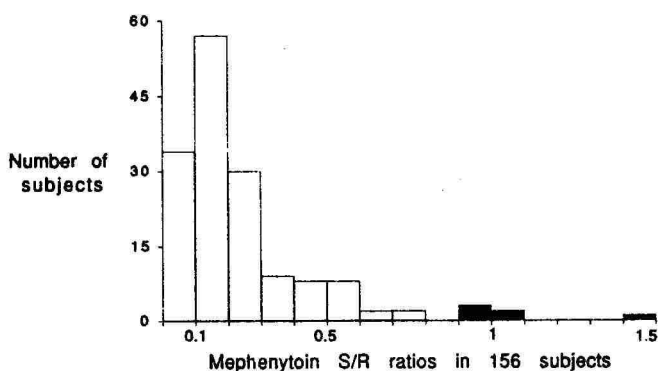
Figure 1. The frequency distribution of the urinary mephenytoin S/R ratio and metabolic ratios of debrisoquine and dextromethorphan in 156 Estonians. The filled columns represent poor metabolisers.

Table 1. Phenotypic traits of EMs and PMs of mephenytoin and debrisoquine/dextromethorphan in 156 unrelated subjects. The median and range are given and the results of urinary recovery of the study drugs are expressed as percentage of dose.

	EM	PM
Mephenytoin		
Nr of subjects	150	6
S/R ratio of mephenytoin	0.17	1.0
	(0.05-0.78)	(0.93-1.44)
Debrisoquine		
Nr of subjects	74	4
Metabolic ratio DEB/4HDEB	0.61	41.1
	(0.08-6.5)	(28-50)
Urinary recovery of DEB	9.4	42
	(1.2-43)	(27-45)
Urinary recovery of 4HDEB	14	1.0
	(3.4-52)	(0.06-1.6)
Total recovery of DEB + 4HDEB	23	43
	(6.1-71)	(28-46)
Dextromethorphan		
Nr of subjects	75	3
Metabolic ratio DXM/DO	0.004	4.2
	(0.001-0.17)	(2.8-5.7)
Urinary recovery of DXM	0.28	4.5
	(0.001-3.5)	(2.6-6.7)
Urinary recovery of DO	22	1.0
	(5.7-75)	(0.9-1.2)
Total recovery of DXM + DO	23	5.0
	(5.7-76)	(3.5-7.9)

Using the antimodes of metabolic ratios 12.6 for debrisoquine [3,6] and 0.3 for dextromethorphan [14], 4 of the 78 Estonians were PM of debrisoquine and 3 of 78 were PM of dextromethorphan (Table 1 & Fig. 1). Thus 4.5% (95% confidence interval 1.2 - 7.8%) were PM of debrisoquine/dextromethorphan. The antimodes used here seem to apply also for this population. The total recovery of parent drug plus metabolite was higher in PM (median 43%), than in EM (23%; p - 0.01) of debrisoquine and the opposite for dextromethorphan (median 5% in PM and 23% in EM; p - 0.005).

None of the subjects were PM of both debrisoquine/dextromethorphan and S-mephenytoin.

Discussion

The finding that 3.9 % of our subjects were PM of mephenytoin and 4.5 % were PM of debrisoquine/dextromethorphan is in agreement with the average prevalence of these phenotypes in other European populations [1].

While the distinction of individual phenotypes is reflected by a more marked difference in the urinary mephenytoin S/R ratio, when using later or longer urine collection times [3,13], this advantage must be weighed with the practical realities in an outpatient setting. Therefore, in our study we chose to later retest 3 potential PM and a later urine sample (24 - 32 h) confirmed the phenotype. Three other potential PM were not available for retesting and we therefore used acidification to confirm the phenotype. Our group has previously shown, that acidification of the 0 - 8 h urine samples from 30 EM of S-mephenytoin increased the S/R ratio 8 - 127 fold, while there was no change in the urine samples of 12 PM (Tybring & Bertilsson, to be published).

The total recoveries of DXM plus DO in our study is somewhat lower than those found previously [14]. The reason for this is not clear, but it is not likely to be the result of incomplete urine collection, as the recoveries of DEB plus 4HDEB are similar [15] or even higher [12], than the values reported in other Caucasian populations.

The greater total recovery in urine the administered dose of DEB in PM subjects is due to a greater excretion of DEB. Despite the small sample size, this difference does not seem to be a chance finding, as it has been reported earlier in a large population [15]. It seems plausible, that in EM subjects of DEB, the debrisoquine hydroxylase forms more of the other metabolites parallel to 4HDEB. Such metabolites are usually not mea-

sured, but have been found to account for 3 to 14% of the dose by Idle et al. in a small study population [10].

The importance of the hydroxylation polymorphism is not only academic. It may result in clinically important consequenses, as the metabolism of several groups of drug appears to cosegregate with the debrisoquine and mephenytoin hydroxylation trait [5,20]. This study has shown, that Estonians are not more prone to the possible diverse clinical effects of the drugs metabolised by polymorphic hydroxylation, than other European Caucasians.

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THE EFFECT OF VARIOUS ANXIOLYTIC/SEDATIVE DRUGS ON THE LOCOMOTOR ACTIVITY OF SLEEP-DEPRIVED MICE.

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Abstract

In the present investigation, the effect of sleep deprivation (SD) on the locomotor activity of mice has been studied. The effect of benzodiazepine (BDZ) receptor agonist diazepam, BDZ receptor antagonist flumazenil, BDZ receptor inverse agonist RO 15-4513 as well as non-benzodiazepine type anxiolytic/sedative drugs buspirone and baclofen on SD-induced changes in the locomotor activity of mice have been also studied.

SD for 24 hours using the platform technique caused significant increase in the locomotor activity.

Administration of diazepam (0.25 and 0.5 mg/kg), flumazenil (5 and 10 mg/kg), buspirone (2, 4 and 8 mg/kg) and baclofen (1 mg/kg) excerted significantly more pronounced inhibitory action on the locomotor activity of SD mice in comparison to control. These data suggest that SD induced a sensitisation of mice to the sedative effects of anxiolytic/sedative drugs. A possible involvement of benzodiazepine receptors in this phenomenon is discussed.

Introduction

Sleep deprivation (SD) produces in laboratory animals several behavioural changes which are reflected by an increased aggressiveness (21), motor activity (2,3,16,17,20,33,45), sexual behaviour (31), anxiety (19,46) and susceptibility to seizures (8,34,42,43,44).

The most widely used technique for SD is platform technique which consists of keeping the animals on a small platform surrounded by water.

The platform technique involves besides its specific action on sleep several other factors of stress such as isolation , immobilization , falling into the water and soaking (16,17) which produce an heavy stress in

laboratory animals (26,27). This experimental model must therefore be considered as a stress model of which SD is one factor (16). In connection with that there has been much discussion about the use of platform technique, in particular about the high level of non specific stress (7,22,26,32). A peculiar feature of this model is that following the period of SD the animal does not fall asleep as could be expected but shows a constant period of wakefulness.

The effects of many drugs known to interfere with central nervous system are different in sleep deprived animals to compare with control animals. Several studies have demonstrated that SD rats show an augmented response to dopaminergic agents as indicated by an intensification of agrressiveness, stereotypy and rearing induced by apomorphine (4,5,6,9,10,11,38,39,40,41). Some works (16,17) claim that excitement which immediately follows SD is related mainly to the hyperactivity of dopaminergic systems, especially D1 receptors.

There is evidence that acetylcholine, norepinefrine, opioids, 5-hydroxytryptamine and other neurotreancmitters are involved in the behavioural changes induced by SD (36,16,17,29,35,36, 25,30).

There are fewer data about the influence of drugs acting at the benzodiazepine receptor on the behavioural changes induced by SD. In some studies neither diazepam nor the benzodiazepine receptor antagonist flumazenil (RO 15-1788) showed different action in SD animals as compared to the controls (16). However, our previous studies revealed a decrease in GABA-stimulated chloride uptake by synaptoneurosomes prepared from the cortex of SD mice ( ). These observations suggest that some changes might occur in GABA-benzodiazepine-barbiturate complex might be involved in SD animals.

Therefore we decided to study the effects of diazepam, BDZ receptor antagonist flumazenil (RO 15-1788) and BDZ receptor partial inverse agonist RO 15-4513 on the changes in the locomotor activity of mice caused by sleep deprivation. In addition to this, the effect of 5-HT_{1A} receptor agonist buspirone and $GABA_B$ receptor agonist baclogfen were also studied.

Materials and methods

Animals-Male albino mice, weighting 25-30g were used throughout the study. Mice were maintained at 20 ± 2 C with water and food ad libitum. All experiments were carried out between 12 a.m. and 2 p.m.

Sleep deprivation. Mice were deprived of sleep using the platform

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technique. Mice were kept on platform (d=3cm) surrounded by water for 24 hours, and were removed from the platform straight before the measurement of locomotor activity.

When drug or saline injections were carried out mice were returned to the platform before measurements.

The measurement of locomotor activity. Locomotor activity was measured in actometer which consisted of individual cages: a cylinder with an inner diameter of 40 cm and 2 photocells located in the walls. Locomotor activity was measured during 60 minutes. Counts were registered after every 15 minutes.

Drugs: The following drugs were used: diazepam, flumazenil (RO 15-1788), RO 15-4513 (Hoffman-LaRoche, Switzerland) , buspirone and baclofen (Ciba Geigy, Basle, Switzerland). All drugs were suspended in saline with a drop of Tween-80. The volume injected was adjusted with saline to 0.1 ml/10 g body weght. All drugs except RO 15-4513 were injected intraperitoneally (i.p.) 30 min prior to test. RO 15-4513 was injected i.p. 5 min prior to experiment.

Statistical analysis

All data were analyzed using one-way analysis of variance Anova. Further statistical analysis was made by Student's t-test.

Results

The effect of sleep deprivation on the locomotor activity of mice is shown in the Table 1.

SD caused significant rise in the locomotor activity of mice which lasted about 60 minutes.

In the low dose (0.25 mg/kg) diazepam did change locomotor activity neither in the control group nor in the SD animals (Table 2).

The higher dose of diazepam (0.5 mg/kg) inhibited locomotor activity in both: control and SD mice. However, the inhibitory effect of diazepam was more pronounced in SD animals. BDZ receptor agonist flumazenil and BDZ receptor inverse agonist Ro 15-4513 in the doses used did not not affect locomotor activity of control animals. However, when flumazenil and Ro 15-4513 were administrered to SD mise these drugs significantly inhibited locomotor activity (Table 3 and 4).
Similar inhibitory effect on locomotor activity in SD mice but noty in control was observed after administration of buspirone and baclofen (Table 5 and 6).

Table 1. The effect of sleep deprivation (SD) on the locomotor activity of mice. Locomotor activity was measured in actometer during 60 minutes. The number of counts was registered after every 15 minutes. The data are mean values ± SEM. The number of animals was 13 in the control group and 10 in SD group. *P<0.05; **P<0.01

	GROUP	C O U N T S
0-15 MINUTES	CONTROL	133.4±21.5
	SLEEP DEPRIVATION	353.5±61.1**
15-30 MINUTES	CONTROL	112.3±22.9
	SLEEP DEPRIVATION	272.4±52.0*
30-45 MINUTES	CONTROL	70.1±19.8
	SLEEP DEPRIVATION	193.1±42.2*
45-60 MINUTES	CONTROL	51.1±19.0
	SLEEP DEPRIVATION	195.1±42.5*
'TOTAL COUNTS	CONTROL	366.9±64.3
	SLEEP DEPRIVATION	1029.3±171.4**
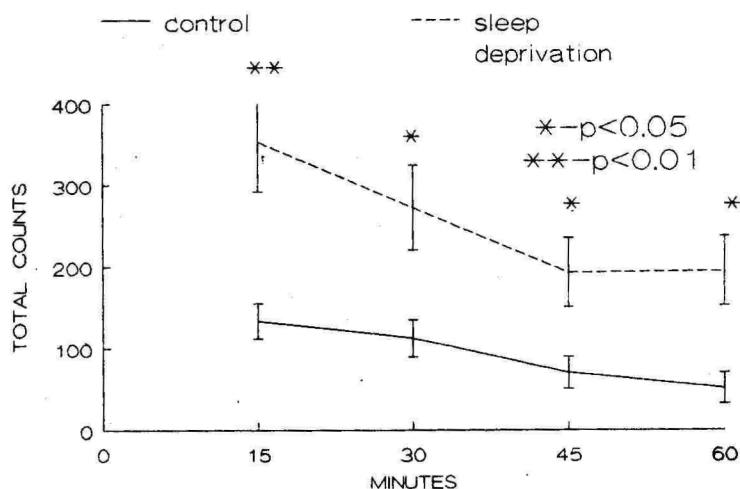
Figure 1. The effect of sleep deprivation on the locomotor activity

Table 2. The effect of diazepam on the changes in the locomotor activity of mice caused by sleep deprivation (SD). The data are mean values ± SEM of groups from 7-8 animals. *-P<0.05, **-P<0.01

Dose (mg/kg)	Group	Counts/60 min	% of inhibition
0.25	CONTROL/SALINE	492.1±82.3	
	CONTROL/DIAZEPAM	313.6±78.8	-57.2%
	SD/SALINE	511.6±119.7	
	SD/DIAZEPAM	419.6±146.8	-22.0%
0.5	CONTROL/SALINE	549.2±69.0	
	CONTROL/DIAZEPAM	331.9±56.9*	-65.9%
	SD/SALINE	788.6±111.9	
	SD/DIAZEPAM	143.4±64.1**	-81.8%
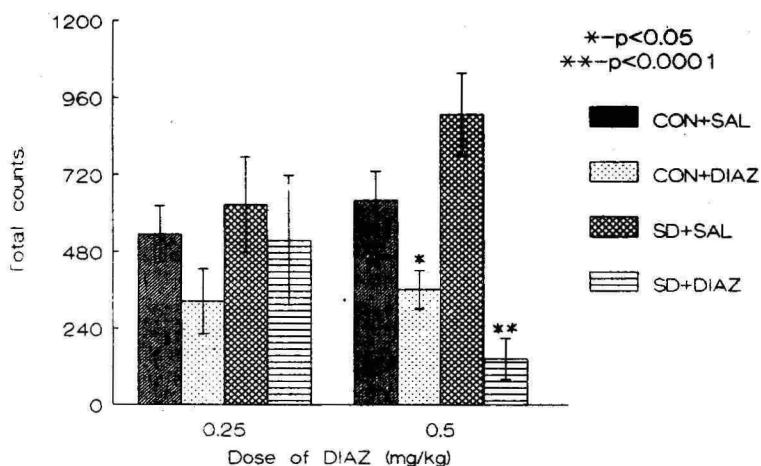
Figure 2. The effect of diazepam on the locomotor activity of mice

Table 3. The effect of flumazenil on the changes in the locomotor activity of mice caused by sleep deprivation (SD).
The data are mean values ± SEM of groups from 10 animals.
* - P<0.005, ** - P<0.001

DOSE MG/KG	GROUP	COUNTS	% OF INHIBITION
5	CONTROL/SALINE	385.3±16.7	
	CONTROL/FLUMAZENIL	310.9±9.1	-19.3%
	SD/SALINE	932.0±49.1	
	SD/FLUMAZENIL	273.4±16.7**	-70.6%
10	CONTROL/SALINE	574.9±22.1	
	CONTROL/FLUMAZENIL	369.0±17.5**	-35.8%
	SD/SALINE	851.5±35.6	
	SD/FLUMAZENIL	405.6±34.6**	-52.3%

Table 4. The effect of RO 15-4513 in the dose 1mg/kg on the changes in the locomotor activity of mice caused by sleep deprivation (SD). The data are mean values ± SEM of groups from 12 animals. *P<0.0001

GROUP	Counts/60 min	% OF INHIBITION
CONTROL/SALINE	425.8±33.2	
CONTROL/RO 15-4513 1MG/KG	55.9±6.3	+7.0%
SD/SALINE	1031.3±33.1	
SD/RO 15-4513 1MG/KG	551.9±26.5*	-46.4%

Table 5. The effect of buspirone on the changes in the locomotor activity of mice caused by sleep deprivation (SD). The data are mean values ± SEM of groups from 7-8 animals. *P<0.05, **P<0.001

DOSE MG/KG	GROUP	Counts/60 min	% OF INHIBITION
2	CONTROL/SALINE	391.6±53.9	
	CONTROL/BUSPIRONE	357.3±87.8	-8.6%
	SD/SALINE	534.6±72.8	
	SD/BUSPIRONE	140.8±19.0**	-73.6%
4	CONTROL/SALINE	530.6±51.3	
	CONTROL/BUSPIRONE	353.4±74.0	-33.3
	SD/SALINE	753.6±145.1	
	SD/BUSPIRONE	159.5±65.6*	-78.8%
8	CONTROL/SALINE	431.8±145.7	
	CONTROL/BUSPIRONE	219.2±94.7	-49.1%
	SD/SALINE	602.2±219.5	
	SD/BUSPIRONE	66.7±25.9*	-88.911%

Table 6. The effect of baclofen in the dose 1mg/kg on the changes in the locomotor activity of mice caused by sleep deprivation (SD). Locomotor activity was measured in actometer during 60 minutes. In this table the total number of counts during 60 minutes is shown. The data are mean values ± SEM of groups from 7-8 animals. *-P<0.05

GROUP	COUNTS	% OF INHIBITION
CONTROL/SALINE	404.6±84.1	
CONTROL/BACLOFEN 1mg/kg	293.3±92.1	-27.4
SD/SALINE	946.6±388.9	
SD/BACLOFEN 1mg/kg	167.6±48.835	-82.3

Discussion

The results of this study indicate that sleep deprivation (SD) causes an increase in the locomotor activity of mice.

In our experiments, BDZ receptor agonist diazepam, BDZ receptor antagonist flumazenil (RO 15-1788) and BDZ receptor partial inverse agonist RO 15-4513 despite of their different action on the locomotor activity in control mice had uniform inhibitory effect on the locomotor activity of SD mice. A substantial number of studies have shown that behavioural effects of these drugs are mediated via central benzodiazepine receptors (FRATTA et al 1987 and 1990).

In animal studies, BDZ receptor agonists and BDZ receptor inverse agonists produce opposite effects on the several behaviours. Although, benzodiazepine receptor antagonist flumazenil given alone does not produce significant behavioural changes in naive animals, it is capable of antagonizing the behavioural effects of both: BDZ receptor agonists and BDZ receptor inverse agonists.

An uniform inhibitory action of these drugs on locomotor activity in SD mice evenin the doses which do not affect locomotor activity in control mice show that benzodiazepine receptor became sypersensitive to these drugs. However an increase in sensitivity of BDZ receptor itself cannot explain observed inhibitory effect of drugs with opposite mechanism of action. It is not excluded that benzodiazepine receptor might exist in several interconvertible states e.g. agonist-state, inverse agonist-state or antagonist-state. Such a possibility has been discussed in many radioligand binding studies. In normal conditions there is a functional equilibrium between several states of BDZ receptor. Stress factors, like SD, induce conversion of BDZ receptor into one particular state, for example, agonist-state. Under these conditions inverse agonist and antagonist at BDZ receptor receptor might act as agonists and exhibit uniform sedative action on the behaviour.

The results of our experiments support the data expressed in the literature (Sallanon et al 1983, Santos and Carlini 1983) that SD causes the sensitisation of animals to the action of serotoninergic drugs. The inhibitory effect of buspirone (acting at the 5-HT receptors) was more pronounced in SD group. The cause of an increased response to serotoninergic drugs seen in SD animals is probably due to the increased sensitivity of postsynaptic serotonin receptors.

Experiments with GABAB receptor agonist baclofen showed that the altered function of GABAB receptors may also have a role in excitement following SD.

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BIOPERIODICAL CHANGES OF ANTIHYPOXIC EFFECT OF NEUROLEPTIC DRUGS IN ALBINO MICE

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Abstract

In long-lasting experiments on albino mice spontaneous resistence to hypoxic-hypoxia, levomepromazine (5 mg/kg) and haloperidole (0.5 mg/kg) induced antihypoxic effect were studied. The motorical activity on one hand and resistance to hypoxia and animals body temperature on the other vary during the year partly in opposite stages. The influence of neuroleptic drugs on these parameters has also seasonal variations. Important role is played by the monoaminergic systems that are the main mechanisms of adaptation to any influence that of drugs as well.

Introduction

Extremal factors' influence on organism finally damages the O_2-regime and metabolism in tissues. In the pathogenesis of nervous disorders a hypoxic component plays important role [4]. Derivatives of gamma-oxybutyric and gamma-aminobutyric acids are known as antihypoxic preparations. Neuroleptics are also used as antihypoxicants. One group of the butyrophenon neuroleptics (haloperidol etc.) are the derivatives of aminobutyric acid as well [11]. The phenothiazin derivatives (levomepromazine) raise anabolic processes and cause so-called "economical state of organism" [9].

At the present time the rhythms of organism's biological reactivity to the physical and chemical factors, pharmacological and toxicological actions included, are well-known [1,2,3,5,7]. This is notable in the bioperiodical changes of the organism's tolerance, the receptors' sensibility to drugs and in the pharmacokinetics of substances [6].

If the experiments are carried out always at the same daytime one can

see seasonal changes of tolerance to chemical compounds. There are known seasonal variations of antihypoxic effect of several drugs [7,8,9]. A clear periodicity emerges in the activity of neuroleptics during the 24 hours. The sedative effect of haloperidol is more notable during darkness, while the animals' motor activity is higher. The reverse of the light regime (12 hours darkness in the daytime) caused the reverse of the effect of the neuroleptic drugs. Haloperidol has the peak of catalepsy in rats during day-time (12.00-14.00). At night the cataleptic effect is minimal. The cataleptic action of neuroleptics has seasonal variation too [2,3,10].

The aim of the present paper is to study the seasonal variations of resistance variations of resistance of the mice in the states of hypoxia and antihypoxic effect of neuroleptics (haloperidol and levomepromazine).

Material and methods

Seasonal experiments were carried out with 450 albino male and female mice with weight 23±0.3 grams. The animals were kept under standard vivarium conditions in an artificial luminescence light (light:dark, 12 hours:12 hours) at temperature 20±2°C, food and water - *ad libitum*. Seasonal rhythm was studied once in two weeks during two years (11.30 a.m.).

Haloperidol (0.5 mg/kg) and levomepromazin (5 mg/kg) were injected i.p. 60 min before the behavioral and antihypoxic tests. Hypoxic-hypoxia was caused by putting the mice into a hermetic chamber (80 cm^2) without CO_2 absorption. The protecting effect of the neuroleptic drugs in the state of hypoxia was determined by the mice's increasing survival period.

In parallel, the changes in motorical, orientation and investigation activity, emotional reactivity according to vocalisation threshold (in volts), agressiveness caused by electric pain irritation and changes in body temperature (measured per rectum) were studied.

Results and discussion

The resistance of intact mice's against hypoxic hypoxia essentially varies during the year (Table 1). It is high in February, March and September, low in July and December. An average mouse survives hypoxia in a 80 cm^2 hermetic chamber for 564±25 seconds. In December the survival period shortens for 2.5 minutes, in March it becomes longer for 1.5 minutes. During these months a remarkable negative correlation of resistance against hypoxia and motorical activity of mice appears.

Table 1. Annual variation of resistance of intact mice to hypoxic-hypoxia and antihypoxic effect of levomepromazine (5 mg/kg) and haloperidol (0.5 mg/kg) 60 minutes after administration. The mean data (M±m) to groups of 10-15 animals.

Months	Duration of life (sec)		
	Intact mice	After administration of	
		Levomepromazine	Haloperidol
I	588 ± 30	1124 ± 113	691 ± 18
II	649 ± 30	1624 ± 82	711 ± 37
III	670 ± 23	1870 ± 136[+]	734 ± 45
IV	527 ± 22	1345 ± 206	770 ± 59
V	644 ± 42	2218 ± 381	656 ± 43
VI	454 ± 24[++]	974 ± 93	671 ± 47
VII	457 ± 30[++]	1266 ± 202	659 ± 75
VIII	541 ± 11	769 ± 135	520 ± 52
IX	645 ± 26[++]	1681 ± 149	750 ± 66[+]
X	618 ± 30	2033 ± 304[+]	645 ± 43
XI	573 ± 19	1272 ± 221	685 ± 47
XII	409 ± 25[++]	1395 ± 297	654 ± 35
Annual mean	564 ± 25	1464 ± 193	671 ± 47

In comparison with annual mean [++]$P \leq 0.01$
[+]$P \leq 0.05$

Levomepromazine (5 mg/kg) prolongs the mice's survival period in the hermetic chamber 2.63 times at an average. Seasonal mean survival period is 3.2 minutes, after administration of levomepromazine over 21 minutes. Seasonal increase of the survival period with levomepromazine varies essentially. The protective effect of the drug is stronger in May and October. The effect is the lowest in August.

The antihypoxic effect of haloperidol (0.5 mg/kg) is less expressed in comparison with levomepromazine. This may depend on different doses and different pharmacological effect. Haloperidol, unlike levomepromazine, has an activating component that raises animals' muscular tone a little. But not less, the antihypoxic effect of haloperidol varies

during the year having peaks in April and September. The lowest antihypoxic action appears in August.

Biological rhythms guarantee the endurance of organism's homeostasis and adaptation in the conditions of internal and environmental changes. An important part is played by the adrenergic and adrenocorticotropic systems that are the main mechanisms of organism's adaptation to any influence, that of drugs as well. Adrenergic system is also connected with the animals' motorical activity.

In animals' antihypoxic resistance their motorical activity plays the determinative role. Animals with high activity are more resistant (Figure 2).

On figure 1 there is given the seasonal dynamics of mice resistance to hypoxic-hypoxia. In most cases higher motorical activity is binded with low resistance against hypoxia, but this negative correlation is only partial.
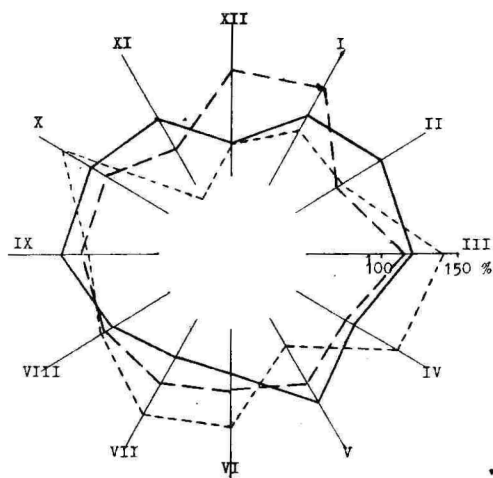
Figure 1. Annual dynamics of the resistance of intact mice to hypoxic-hypoxia (——), to electrical pain stimulation (— —) and motor activity (- - - - -) [%-s of annual means.
Annual means:
Resistance of intact mice to hypoxic-hypoxia 562.0±25 sec, to electrical pain stimulation (summarized) 52.9±3.9 volts, their motor activity 258.6±19 impulses.
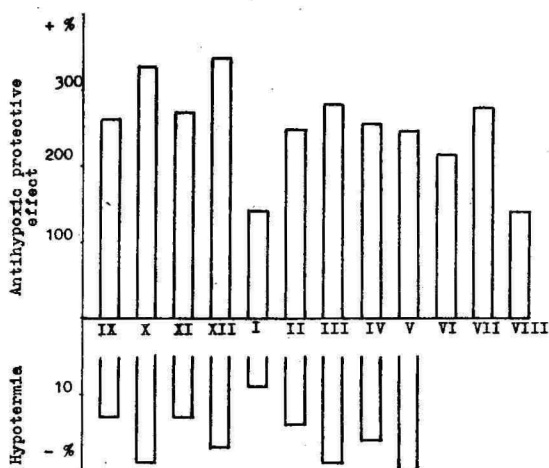
Figure 2. Seasonal dynamics of the levomnepromazine (5 mg/kg) induced antihypoxic protective effects and hypothermia in mice 60 min after administration (% of annual means).

Annual means:

Antihypoxic protective effect of levomepromazine in mice is +1263% of the resistance of intact mice to hypoxic-hypoxia and -14% of hypothermia.

Comparing the mice's resistance to hypoxia with the threshold caused by electric pain irritation one can see parallelism. During spring and autumn months the mice's resistance to electric irritation as well as hypoxic action are higher. These data are in correlation with content and speed of metabolism of dopamine in mice brain [6].

The neuroleptic drugs' antihypoxic protective effect is to a great extent caused by hypothermia over the serotoninergic system blockade (Figure 2).

The studied drugs, especially levomepromazine, have the serotonin blockading effect that leads to the increase of the amount and metabolism speed of serotonin.

In June and December the resistance of intact mice to hypoxia is at its lowest (Table 1), while the content of serotonin in the whole brain has its maximum peak [9].

Conclusions

1. The intact mice's motor activity, body temperature and resistance to hypoxia vary during the year, partly in opposite stages. The neuroleptic drugs' influence on these parameters has also seasonal variations.

2. During the year haloperidol and levomepromazine have their highest effect on an axis spring-autumn months.

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