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Effect of Glutamate Receptor Antagonists on Suckling-Induced Prolactin Release in Rats

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The aim of the present study was to investigate the role of endogenous excitatory amino acid receptors in suckling-induced prolactin (PRL) elevation. Glutamate is known to be the dominant excitatory neurotransmitter and may act simultaneously on different glutamatergic receptor subtypes. MK-801 (dizocilpine) is a noncompetitive antagonist of the *N*-methyl-D-aspartate (NMDA), while GyKI 52466 is an antagonist of the *R*,*S*-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)/kainate receptor subtypes. Using the combination of the two receptorsubtype antagonists, we tested the hypothesis that parallel blockade of more than one subtype is more effective. Low-dose MK-801 (0.033 mg/kg) had no effect on suckling-induced PRL elevation after 4 h of separation. When injected alone, 10 mg/kg of GyKI 52466 was also ineffective, but in combination with low-dose MK-801 it efficiently diminished the suckling-induced PRL elevation while lactation proceeded. The same dose of GyKI 52466 combined with 0.2 mg/kg of MK-801 (a combination that in other studies was able to block the foot-shock-induced PRL elevation) was more effective. Simultaneous blockade of the two ionotropic glutamate receptors with 0.2 mg/kg of MK-801 and 10 mg/kg of GyKI 52466 caused a decline in plasma PRL concentration of continuously suckling mothers. We conclude that the endogenous glutamatergic system has an important role in suckling-induced PRL elevation and in the maintenance of constantly high PRL levels in lactating mothers. Furthermore, the NMDA and AMPA/kainate receptor subtypes can interact with each other in this process.

Key Words: Excitatory amino acids; MK-801; dizocilpine; GyKI 52466; lactation.

Introduction

Several lines of evidence suggest that glutamate is the dominant excitatory neurotransmitter in neuroendocrine regulations *(1,2)*. Glutamate exerts its action through distinct receptor groups, including the voltage-gated *N*-methyl-D-aspartate (NMDA)–preferring ionotropic and the non-NMDA-preferring ionotropic excitatory amino acid (EAA) receptor family (among others the *R*,*S*-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid [AMPA]/kainate coreceptors) as well as several subtypes of metabotropic receptors *(3)*. There is ample evidence that many neurons express simultaneously more than one type of glutamate receptors *(4,5)*. The AMPA/kainate receptor is responsible for fast information transfer while the NMDA receptor is only operative when the postsynaptic membrane is independently depolarized by another receptor, such as by AMPA/kainate receptor *(6)*. For this reason, modulation of more than one receptor may be needed to markedly modify physiologic responses mediated by glutamate.

Receptor antagonists preferring different glutamate receptor families are now available to study the role of this endogenous neurotransmitter. For example, MK-801 (dizocilpine) is a noncompetitive antagonist of the NMDA receptor family, while GyKI 52466 is a negative allosteric modulator of the AMPA/kainate receptors *(7,8)*.

The suckling-induced release of prolactin (PRL) is a widely studied neuroendocrine reflex *(9,10)*. Its neural afferentation is known to be composed of classic sensory as well as central autonomic neuronal networks terminating at the hypothalamus that activate the humoral efferent side, which results in the release of PRL from the anterior lobe of the pituitary gland *(11,12)*. Separation of the mothers from their pups for 4 h results in a fall of plasma PRL levels. When they are reunited, plasma PRL level begins to rise within a few minutes but falls again when nursing is terminated *(13,14)*.

EAAs appear to be potent modulators of PRL secretion in male and cycling female rats *(2)*. Previous studies have shown that in lactating rats NMDA and kainate do not stimulate PRL release, which could be owing to the high PRL levels in these animals *(15–17)*. When the baseline PRL secretion was low, both NMDA and kainate stimulated PRL release after intracerebroventricular (icv) application *(17)*.

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Moreover, in lactating animals systemic administration of either drug inhibited PRL secretion *(17)*, perhaps owing to the parallel pituitary-adrenal system activation. According to Parker and Crowley *(18)*, an AMPA antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), injected into the third ventricle abolished the suckling-induced increase in plasma oxytocin and PRL levels up to 45 min. These results suggest that glutamatergic influence may operate and have physiologic significance in the regulation of those pituitary hormones (oxytocin and PRL) that are released owing to suckling stimulus.

It is well known that the pituitary-adrenal system plays an important role in the initiation and maintenance of lactation in rats *(19)*. Parallel with PRL release, suckling directly stimulates the secretion of adrenocorticotropic hormone (ACTH) and corticosterone *(20)*. When exposed to different stressors (ether exposure, restraint, noise, endotoxin), lactating rats respond with attenuated release of PRL and corticosterone *(21–23)*. Moreover, in mothers kept together with their pups, corticosterone levels rise and the plasma PRL levels decrease simultaneously after stress *(21)*. Because of the possible side effects of the glutamate receptor antagonists, we tested whether the administration of the drugs is a stress *per se* causing ACTH/corticosterone elevation and a consecutive decline in PRL release.

With these restrictions, the possible role of endogenous EAAs, acting on different receptor subtypes, in the neural control of PRL release from the adenohypophysis during suckling stimulus was investigated in freely moving rats.

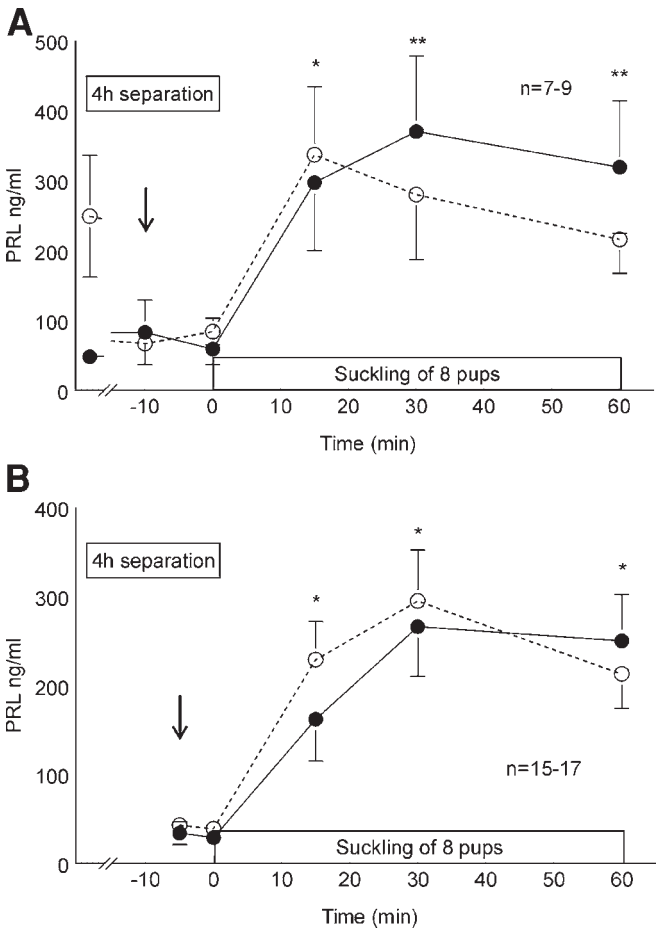

Fig. 1. Effect of iv administration of (**A**) 0.033 mg/kg of MK-801 (NMDA antagonist) or (**B**) 10 mg/kg of GyKI 52466 (AMPA/kainate antagonist) on suckling-induced PRL elevation in lactating mother. (○) Control, saline-treated animals; (●) PRL data from MK-801- or GyKI-treated mothers. Both treatments were ineffective on the suckling-induced PRL elevation.

Results

Consistent with previous observations, the PRL levels fell to a very low level during the 4-h separation period *(21,24)*. The suckling itself caused a significant PRL elevation already at 15 min, and PRL remained elevated during the observed period. When we tested the same animal 2 d later, the suckling-induced PRL release or the constantly high PRL fluctuation was the same independently of the previous treatment.

Different doses of MK-801 were injected intravenously 10 min before replacement of the pups. Within 10 min neither dose influenced the low basal PRL levels (4 h after separation, before returning the pups). The high doses (0.5, 0.25, and 0.1 mg/kg) caused a remarkable locomotor side effect and the mothers did not nurse; we do not present these data because of the lack of suckling. The smaller dose (0.033 mg/kg) was without any locomotor side effect but also did not influence the PRL elevation caused by suckling (Fig. 1A).

A dose of 10 mg/kg of GyKI 52466 injected 5 min before the beginning of the suckling did not influence basal PRL levels within 5 min. This dose remained ineffective through the whole experiment (Fig. 1B).

To test the idea that in vivo glutamate activates more receptor subtypes simultaneously, we combined the antagonists of the two receptor subtypes. GyKI 52466 (10 mg/kg) was combined with low-dose MK-801 (0.033 mg/kg), and this combination diminished the suckling-induced PRL elevation at 15 and 30 min (Fig. 2A). The effect disappeared by the end of the experiment. We also tested a higher dose of MK-801 (0.2 mg/kg) in combination with 10 mg/kg of GyKI 52466 because the muscle relaxant side effect of GyKI 52466 was expected to counteract the MK-801 locomotor side effect *(25)* and the mothers were suckled. Furthermore, in our previous experiments this combination diminished the foot-shock stress–induced PRL elevation *(26)*. This combined treatment caused only minimal behavioral changes; the females did not circulate and the pups were suckling. Moreover, this combination (0.2 mg/kg of MK-801 and 10 mg/kg of GyKI 52466) completely abolished the suckling-induced PRL elevation at 15 and 30 min (Fig. 2B). The parallel corticosterone data show no changes (Table 1; $p = 0.858$).

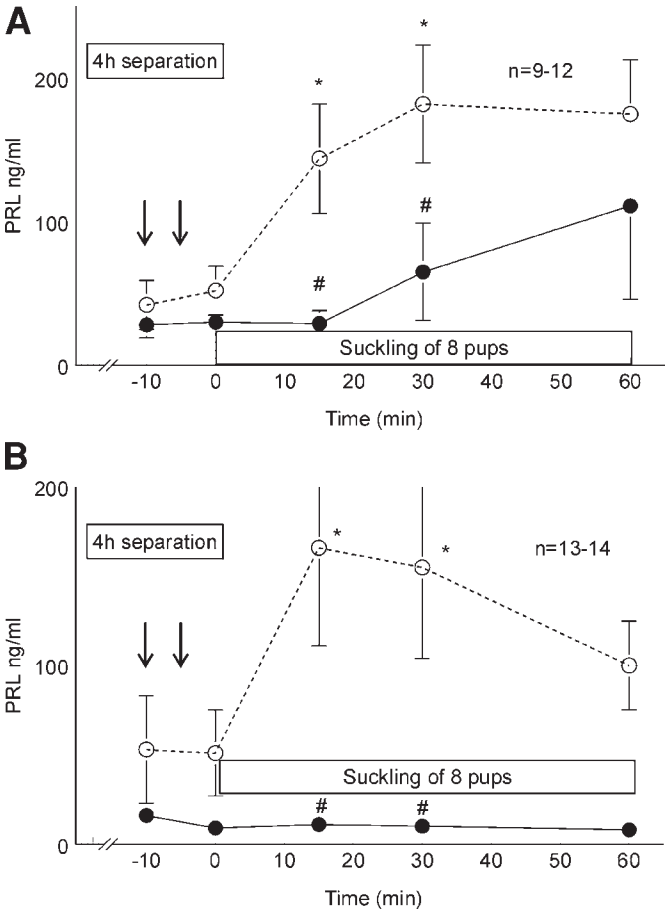

Fig. 2. Effect of combined treatment with (**A**) 0.033 mg/kg and (**B**) 0.2 mg/kg of MK-801 and 10 mg/kg of GyKI 52466 on PRL elevation caused by suckling. (○) Control mothers; (●) data from treated animals. The combined treatment significantly lowered the suckling-induced PRL elevation. *$p < 0.05$ and **$p < 0.01$ significant difference from 0-min values; $^{\#}p < 0.05$ significant difference from saline-treated animals.

Table 1
Plasma Corticosterone (pmol/mL)
Changes in Lactating Mothers (n = 6–13) After 4 h of Separation Treated Intravenously with Either Saline or 0.2 mg/kg of MK-801 and 10 mg/kg of GyKI 52466

	−10 min	0 min	15 min	30 min	60 min
NaCl	827 ± 219	762 ± 188	1101 ± 214	1236 ± 122	1680 ± 136
MK + GyKI	471 ± 124	1016 ± 118	1059 ± 122	1007 ± 45	1396 ± 218

When the offspring suckled in the afternoon without prior separation, the plasma level of PRL was not very high and fluctuated (Fig. 3A open circles, and 3B). The combined treatment with the NMDA and AMPA/kainate receptor antagonists lowered average PRL level (Fig. 3A closed circles, and 3C). The corticosterone levels in the same samples were

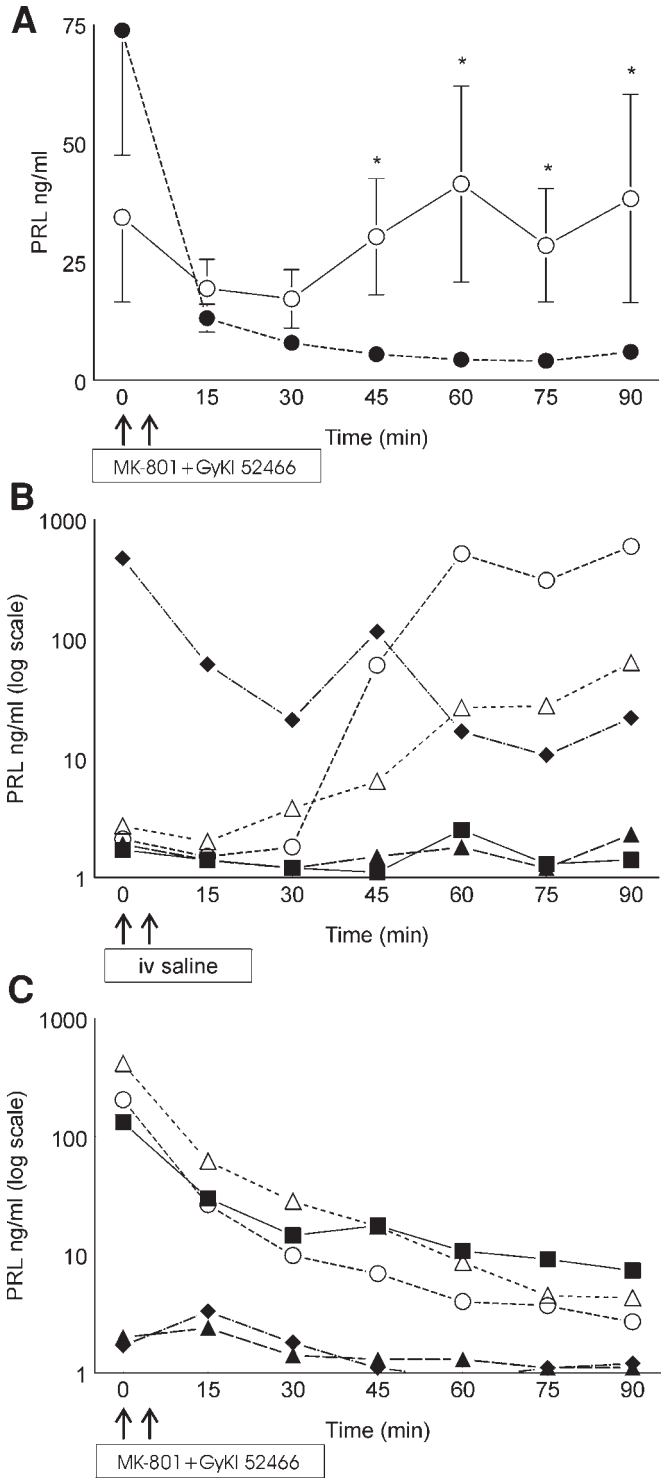

Fig. 3. Suckling in afternoon without prior separation of offspring (continuous suckling). Mother rats were treated with either saline (open circles in [**A**] and all symbols in [**B**]) or a combination of 0.2 mg/kg of MK-801 (at 0 min) and 10 mg/kg of GyKI 52466 (at 5 min) (closed circles in [**A**] and all symbols in [**C**]). In each experiment, half of the animals received one of the treatments and 2 d later the opposite treatment. We examined 32 rats and show mean ± SEM values in (A) and data from 5 representative animals in (B) and (C), with identical symbols in (B) and (C) denoting the same animal. PRL data are shown on linear (A) or logarithmic scale (B,C). The treatment effect was significant between 45 and 90 min. *$p = 0.004$.

Table 2
Plasma Corticosterone (pmol/mL) Changes in Continuously Lactating Rats (n = 10)
Treated Intravenously with Either Saline or 0.2 mg/kg of MK-801 and 10 mg/kg of GyKI 52466

	0 min	15 min	30 min	45 min	60 min	75 min	90 min
NaCl	1050 ± 132	980 ± 111	860 ± 119	1121 ± 257	1306 ± 216	1383 ± 267	1387 ± 280
MK + GyKI	1082 ± 274	977 ± 119	1337 ± 151	1752 ± 233	2056 ± 372	1826 ± 269	1724 ± 233

high and were not affected by the glutamate receptor antagonists (Table 2; $p = 0.183$; between 45 and 90 min: $p = 0.1$).

Discussion

The present results demonstrate that parallel inhibition of NMDA and AMPA/kainate glutamate receptor subtypes effectively blocks suckling-induced phasic PRL release, and that the same treatment is also effective in mothers that persistently nurse their litters. This inhibitory effect persists at least 30 min following initiation of suckling stimulus but cannot be detected 48 h later when tested on the same rats.

We used two glutamate receptor antagonists (MK-801 as NMDA antagonist, and GyKI 52466 as AMPA/kainate antagonist), that are known to enter the brain after systemic administration *(7,8).* Therefore, their site of action can be either at the periphery or in the central nervous system. They are readily soluble in physiologic saline, which makes them suitable for iv application. One of the problems we faced is that MK-801 produces some locomotor activation of the rats in a dose-dependent manner *(27,28).* According to Iversen et al. *(29)*, doses of only >0.3 mg/kg are required to produce profound behavioral changes such as head weaving, body rolling, and hyperlocomotion. In our hands, mother rats injected with a moderate to high dose (>0.033 mg/kg) of MK-801 showed signs of disturbances of motor coordination *(30,31).* GyKI 52466 was found not to be markedly stressful on its own, and from pharmacologic experiments in the literature a dose of 10 mg/kg was considered effective on synaptic transmission *(32).* The effects of GyKI 52466 are known to develop fast and last only for a relatively short time; therefore, it was administered only 5 min before replacement of the pups *(8).* Because behavioral side effects of these drugs may disrupt nursing behavior, we followed with special attention the nursing behavior of the mothers and used only the data obtained from appropriately nursing animals (we rejected the data from experiments using 0.5, 0.25, or 0.1 mg/kg of MK-801). In 1993, Hauber and Andersen *(25)* found that blockade of non-NMDA glutamate receptors (4.8 mg/kg of GyKI 52466 intraperitoneally) antagonized the behavioral stimulant effects of an NMDA receptor blockade (0.08 mg/kg of MK-801, intraperitoneally). We also combined the NMDA and non-NMDA receptor blocker, and in our circumstances, mothers treated with 0.2 mg/kg of MK-801 and 10 mg/kg of GyKI 52466 did not move around and the pups were able to suckle.

PRL is a hormone involved in the control of many functions, from osmoregulation in fishes to lactation in mammals. In mammals, suckling induces a release of PRL parallel to an oxytocin-driven milk ejection reflex *(33).* During persistent suckling, plasma PRL and corticosterone are constantly high. It has been shown previously that plasma PRL levels of mother rats are sharply diminished following separation of the pups from them *(21,24).* Administration of MK-801 and/or GyKI 52466 did not affect the very low basal levels of PRL in mothers deprived of their pups for 4 h. This finding is consistent with the work of Pechnick et al. *(7,34)* and our previous results *(26),* which showed that the glutamate antagonists did not influence basal PRL levels in male as well as in cycling female rats.

The lowest dose (0.033 mg/kg) of MK-801 alone had no effect on suckling-induced PRL release, but higher doses could not be used alone because they disrupted lactation. Because of this locomotor side effect of MK-801, the role of the NMDA receptors *per se* in developing suckling-induced PRL values cannot be evaluated.

GyKI 52466 (10 mg/kg) *per se* was also ineffective on suckling-induced PRL release. This later finding is in contrast with that of Parker and Crowley *(18)*, who found that the AMPA antagonist CNQX injected into the third ventricle abolished the suckling-induced increase in plasma PRL levels. The reason could be that we tried to find a submaximal dose, which could potentiate the effect of the NMDA antagonist. Therefore, we used 10 mg/kg of GyKI 52466, which is far below the LD_{50} (300 of mg/kg), and it does not markedly decrease motility *(8).* Experiments with higher doses would be needed to test whether glutamate acting through AMPA/kainate receptors alone *(18)* is able to influence PRL release in lactating rats.

GyKI 52466 in combination with a low dose of MK-801 diminished the suckling-induced PRL elevation. This effect disappeared by 60 min, which could be owing to the short half-life of the GyKI compound *(8).* The same dose of GyKI 52466 combined with 0.2 mg/kg of MK-801 (a combination that was able to block the foot-shock-induced PRL elevation *[26]*) was more effective, and in this combination

the side effects of the two drugs neutralized each other *(25)*. Czuczwar et al. *(35)* established that in mice the blockade of more than one subtype of glutamate receptors (2.5 mg/kg of GyKI 52466 and 0.2 mg/kg of MK-801) leads to a more pronounced anticonvulsive effect when compared with the effect of blockade of an individual receptor subtype. In our previous studies of foot-shock-induced PRL release in rats, we also observed similar findings *(27)*. We can conclude that in the neuronal pathways of suckling-induced PRL release, there are also glutamatergic synapses, and both NMDA and AMPA/kainate receptors take part in the transmission process. Presumably either agonist alone can play the role of the stimulatory transmitter, but under physiologic conditions both receptors are activated. The distribution of these two glutamate receptor subtypes is only partially overlapping. When they are on the same cells, the possible sequence of events is that glutamate first opens the AMPA/kainate receptors and Na^+ enters into the cell. The depolarization eliminates the Mg^{2+} ion block of NMDA receptors so that Ca^{2+} can also enter the neuron and lead to a more profound activation of the cell. Although prior activation is needed for the opening of the NMDA receptor by glutamate, in some brain areas the blockade of the AMPA receptors is not enough to block the glutamate effect, because other receptor activation may precede the NMDA receptor activation. Our data demonstrate that these two glutamate receptor subtypes can act synergistically in a complex system, where it seems likely that the two receptor subtypes may reside on the same as well as on different neurons and their interaction may manifest at a network level.

In our circumstances in constantly suckling animals, the PRL levels were higher than in nulliparous animals and fluctuated markedly. Because of the aforementioned individual fluctuations, we used the animals twice and compared their PRL values after saline and antagonist treatments. When the mothers received combined glutamate receptor antagonist treatment, PRL levels started to decline and the fluctuation was abolished. This suggests that glutamate acting on NMDA and non-NMDA receptors plays a role in the maintenance of the fluctuating high PRL levels in suckling animals as well.

One can hypothesize that our drug treatment induces nonspecific stress because of the side effects and a stress can block the PRL elevation in lactating rats with a concurrent corticosterone elevation *(21)*. Therefore, we also measured corticosterone from the same plasma samples. We found that those treatments, which effectively diminished suckling-induced PRL response or affected the constantly high PRL concentration in mothers without separation of them from their pups, did not significantly change the corticosterone values. Thus, we suggest that the stressful side effect is negligible.

The drugs used in the present study traverse the blood-brain barrier and they can act either peripherally or centrally. Although there are glutamatergic receptors in the periphery *(36)*, it seems more likely that EAAs act through central mechanisms *(17,18),* because CNQX was effective throughout icv application. NMDA receptors are present in the hypophysis and in the hypothalamus and are able to enhance PRL release in vitro from isolated adenohypophysis and hypothalamus *(37,38)*. These data suggest that direct modulation by glutamate at the level of the anterior pituitary is also possible. Presumably glutamate can influence the suckling-induced PRL release at the level of the hypothalamus as well *(2)*.

In summary, we suggest that the regulatory pathway mediating suckling-induced pituitary PRL release and the maintenance of constantly high PRL levels in lactating mothers may involve glutamatergic neurons. Furthermore, the two different glutamate receptor subtypes—NMDA and AMPA/kainate—can interact with each other in this transmission process.

Materials and Methods

Animals

Primiparous lactating rats of the Sprague-Dawley strain weighing 250–350 g were used. After parturition, animals were housed in single cages together with eight pups at 23 to 24ºC and 50–60% humidity with a 12-h light/dark cycle (lights on at 6:00 AM, off at 6:00 PM). On the d 3–7 postpartum, rats were implanted with venous cannulae. Animals were given rat chow and tap water ad libitum. For iv injections and blood sampling, a silicone-tipped polyethylene cannula was implanted into the right jugular vein under ether anesthesia 2 d prior to the experiments. The animals were handled in accordance with regulations set by the Hungarian Council for Animal Care.

Drugs

GyKI 52466 (1-[4-aminophenyl]-4-methyl-7,8-methylenedioxy-5H-2,3- benzodiazepine hydrochloride, an AMPA/kainate antagonist [mol wt of 330]; a kind gift from Dr. I. Tarnawa, Institute of Drug Research, Budapest, Hungary) and MK-801 ([+]-dizocilpine hydrogen maleate; SIGMA-RBI, Budapest, Hungary; a noncompetitive NMDA receptor antagonist) were dissolved in 0.9% NaCl.

Experimental Procedures

For examining the suckling-induced PRL release, the mothers were separated from their pups at 9:00 AM immediately after taking the first blood sample. At 1:00 PM, 10 min (MK-801) and/or 5 min (GyKI 52466) after the drug injections, mothers received their pups. Blood samples were collected immediately before drug injection (–10 or –5 min) and before pup replacement (0 min) and at 15, 30, and 60 min.

During permanent suckling, iv injections of 0.2 mg/kg of MK-801 (0 min) and 10 mg/kg of GyKI 52466 (5 min) were given to lactating mothers at about 1:00 PM, and blood samples (0.3 mL) were collected at 0, 15, 30, 45, 60, 75, and

90 min. In both cases, half the animals received iv physiologic saline (1 mL/kg, control group). The same animals were used again 2 d later but were given the opposite treatment. Blood samples were centrifuged and plasma was stored on –20ºC until hormone measurement.

The behavior of mother rats was videorecorded, and data were used only from animals with good nursing behavior.

Hormone Assay

Plasma PRL concentrations were measured by radioimmunoassay (RIA) using materials from National Institute of Diabetes and Digestive and Kidney Diseases sent by Dr. A. Parlow (Torrance, CA); the limit of detection was 2 ng/mL of plasma in terms of PRL-RP-2. Intra- and interassay coefficients of variation were 9 and 15%, respectively.

Plasma corticosterone was measured with a RIA using ^{125}I-labeled corticosterone and a specific antiserum as previously described *(39)*. The sensitivity of measurements was 0.1 pmol.

Statistical Analysis

Statistical analysis was performed by repeated-measures analysis of variance followed by Tukey HSD test for multiple comparison, using logarithmic transformation of data and the STATISTICA software (StatSoft, Tulsa, OK). Data are presented as the mean ± SEM (except Fig. 3B,C).

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