Further Evidence for the Role of Nitric Oxide in Maternal Aggression: Effects of L-NAME on Maternal Aggression towards Female Intruders in Wistar Rats

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Short title: The Effect of L-NAME on Maternal Aggression in Wistar Rats

Summary

It has been shown that nitric oxide (NO) increases aggression in male mice, whereas it decreases aggression in lactating female mice and prairie voles. It has been known that aggression can be exhibited at different levels in rodent species, strain or subtypes. The aims of this study were to investigate the proportion of aggressiveness in Wistar rats, the effect of intraperitoneally administered nonspecific nitric oxide synthase (NOS) inhibitor L-NAME (N-G-nitro L-arginine methyl ester) on maternal aggression towards female intruders, and whether these effects are due to NO production or not. Rats were given saline intraperitoneally on the postpartum Day 2 and aggression levels were recorded. The same rats were given 60mg/kg L-NAME or D-NAME (N-G-nitro D-arginine methyl ester) on the postpartum Day 3 and their effects on aggression levels were compared to saline. L-NAME did not cause any differences in the total number of aggressive behaviour, aggression duration and aggression intensity while it reduced the proportion of aggressive animals. Also, the latency of first aggression was significantly increased by L-NAME. In the D-NAME group, however, no significant change was found. Our results have shown that L-NAME reduces maternal aggression towards female intruders in Wistar rats through inhibition of NO production. These results suggest that the role of NO in offensive and defensive maternal aggression seems to share neural mechanisms.

Key words: maternal aggression; nitric oxide; N-G-nitro L-arginine methyl ester; Wistar rat

1. **Introduction**

Lactating female mammals exhibit an aggressive behavior, which is called maternal aggression, to protect their pups towards male or female intruders to the nesting area (Erskine *et al.* 1980, Oliver and Young 2002). Temporarily increased aggression in lactating female is an outstanding feature only during the first two or three weeks after delivery. After the third week, it declines and later disappears even the lactation continues (Erskine *et al.* 1980, Gandelman and Simon 1980, Siegel *et al.* 1983, Svare and Gandelman 1976). Although changes in several hormone and neurotransmitter (i.e. oxytocin, serotonin and dopamine) contents within the brain during this period have been shown, the underlying mechanisms still remain unclear (Consiglio *et al.* 2005, Giovenardi *et al.* 1998, Insel 1986, Nelson and Trainor 2007, Russel and Leng 1998, Svare 1990).

The number of studies to understand the role of NO in behavior has increased since it was determined that nitric oxide (NO) plays role in many physiological and pathological processes as a neuronal messenger or neurotransmitter. The production of NO is catalyzed by NO synthase enzymes (NOS) (Moncada and Higgs 1993). There are at least three NOS isoenzymes which are extensively found in brain tissue: Type I (neuronal NOS; nNOS) in neurons, Type II (inducible NOS; iNOS) in glia cells and Type III (endothelial NOS, eNOS) in endothelial cells (Lincoln *et al.* 1997, Palmer *et al.* 1987).

The nNOS enzyme has a modulating role on male aggression in mice. When the production of NO is blocked via selective deletion of the nNOS gene ($nNOS^{-/-}$) or treatment with a specific nNOS inhibitor, 7-nitroindazole aggression increases (Demas *et al.* 1997; Nelson *et al.* 1995, Nelson and Trainor 2007). Additionally, as wild-type mice reduces aggression in response to submissive displays made by intruders, male $nNOS^{-/-}$ mice continues aggressive behavior in spite of submissive displays (Demas *et al.* 1997, Nelson and Trainor 2007). In contrast, $eNOS^{-/-}$ knockout male mice show dramatic decline in aggression

(Demas *et al.* 1999). On the other hand, it seems that the role of NO in maternal aggression is different than male aggression, at least in mice and prairie voles. It has been suggested that NO increases maternal aggression, whereas its blockade reduces attacks to the intruder in mice and prairie voles (Gammie *et al.* 2000a, Gammie *et al.* 2000b, Nelson *et al.* 1995, Nelson and Trainor 2007).

Maternal behaviors have some differences between mice and rats or laboratory and wild-type mice or more interestingly virgin and primiparous mice (Blanchard *et al.* 2003, Oliver and Young 2002, Russel and Leng 1998). It is possible that there are also some differences between the species, strains and even subtypes in terms of maternal aggression (Blanchard *et al.* 2003, Russel and Leng 1998). To claim the effectiveness of a drug on maternal aggression very definitively, therefore, it should be studied on different species, strains and subtypes. Some subjects may display no aggression in any circumstances, so they have been accepted as nonaggressive in some studies (Svare 1981, Brain 1990, Gammie and Nelson 2001). In a study, the proportion of non-aggressive animals has reported as 42% (n=14) (Gammie and Nelson 2001). Another disputatious matter related to maternal aggression is about the type of this aggression, i.e. is it offensive or defensive? Some studies have suggested that the intruder sex affects the type of this aggression and its levels (Svare 1981, Brain 1990, Oliver and Young 2002).

Almost all previous studies which attempted to investigate the role of NO in maternal aggression used mice or prairie voles. There is only one study which investigated the effect of NO on maternal aggression in rats (Service and Woodside 2007). In the latter study, it has been shown that administration of N-G-Nitro-L-Arginine Methyl Ether (L-NAME), a NO synthase inhibitor, into the third ventricle or the medial preoptic area (MPOA) disrupts maternal aggression. In this study and in many others, all subjects have randomly assigned to different groups, called between subject comparisons, regardless of whether they have

exhibited maternal aggression to a male intruders naturally or not. They were compared with each other in terms of drug effects. In these groups, some subjects would be non-aggressive in all test conditions naturally. For eliminating this type of effect and genetic/environmental effects, we thought that the more appropriate study design would be a comparison within subjects, which compare measured variables in the pre- and post-treatment periods for each subject, instead of comparing between subjects.

In the present study, we aimed to investigate 1) the proportion of maternal aggression in Wistar rats, 2) the effects of intraperitoneally administered L-NAME, a nonspecific NOS inhibitor, on maternal aggression towards female intruders with more proper study design and 3) the effects of i.p. administered D-NAME, an ineffective enanthiomer of L-NAME, on maternal aggression and thereby whether the effects of L-NAME are due to NO production.

2. Methods

2.1. Animals and housing

38 female Wistar rats of 70-90 days old that weigh 242±23g were used in this study. Animals were maintained in standard polycarbonate cages at ambient room temperature, and the light/dark cycle of room was set at 14/10 h. The floor was covered with wood shavings, which were used as bedding and nesting material. Food and water were provided ad libitum. Each female rat was housed with a stud male rat in the same cage and after the beginning of pregnancy; male rats were removed from the cages. All efforts were made to minimize animals' pain and discomfort. Experimental procedures were conducted with a government approval and according to local guidelines for the care and use of laboratory animals.

2.2. Drugs and doses

In this study, the effects of N-G-Nitro L-Arginine Methyl Ester hydrochloride (L-NAME), a nonspecific NOS inhibitor, and its ineffective enanthiomer N-G-Nitro D-Arginine

Methyl Ester hydrochloride (D-NAME) were investigated on maternal aggression. Animals were randomly divided into two groups as L-NAME (n=20) and D-NAME (n=18) groups. As treatment control, 0.5 ml intraperitoneally (i.p.) saline was given to lactating rats and basal aggression levels were measured in both groups on Day 2. A dose of 60 mg/kg i.p. L-NAME or D-NAME, which can be considered as avarage effective dose in pharmacological studies were applied to animals on Day 3 (Uzbay *et al.* 1997, Uzbay and Kayir 2003, Yildirim and Marangoz 2004).

2.3. Behavioral test

Animals were tested for aggressive behavior using the resident-intruder paradigm, a well-characterized valid model of maternal aggression. The date of birth was considered postpartum Day 0. Aggression test was applied to the lactating female rat on postpartum Day 2 and Day 3, because rats are maximally aggressive in the first lactation week towards female intruders and attack behavior is attenuated by the second lactation week (Haney *et al.* 1989). Tests were done between 08:00 and 12:00 hours A.M. on test days. On Day 2, i.p. saline was given to the resident rat 30min. before the experiment. The pups were removed from the home cage 3min. before the aggression test to prevent any harmful situation (Service and Woodside 2007, Siegel *et al.* 1983). Thirty minutes after saline injection an intruder female rat was placed into the home cage and then the aggressive behaviors of the resident rat were observed for 20 minutes. To avoid any effects of contamination by intruder's odorant or familiarity, the cages were cleaned before test days and a different female rat was used in each test as the intruder. All intruders were selected among young virgins and their body sizes were smaller than lactating mothers. None of intruders was aggressive towards resident female rats.

Aggressive behaviors exhibited by lactating rat towards the intruder rat were observed and recorded according to the following parameters (Svare *et al.* 1981);

A. **The latency to the first aggressive behavior:** Time of first aggressive attack or posture exhibited towards the intruder. If there was not any aggressive behavior, total test time (1200 seconds) was used as data of the latency to the first aggressive behavior.
B. **The number of aggressive behaviors:** The number of aggressive behaviors which was exhibited towards the intruder rat.
C. **The total duration of aggressive behaviors:** Total amount of time for aggressive behaviors towards the intruder rat.
D. **The intensity of aggressive behavior:** Intensity of aggressive behavior exhibited by the resident rat was scored as: No aggressive manifestations (0); Intermittent mild aggressive posture or attack towards the intruder, no vocalizations (1); Intermittent intensive upright aggressive posture or attack or boxing with the intruder, vocalizations, but no biting or continuous fighting (2); Continuous fighting or attempts to bite the intruder rat, loud vocalizations (3).

On postpartum Day 3, the same test was repeated with L-NAME or D-NAME applications in L-NAME and D-NAME groups, respectively. The animals which did not exhibit any aggressive behavior by application both saline and L-NAME or D-NAME were accepted as nonaggressive.

2.4. Statistical Analysis

Behavioral measurements, including the latency to first aggressive behavior (seconds), total number of aggressive behaviors, total duration of aggressive behaviors (seconds) and the intensity of aggressive behaviors were recorded as data. Descriptive statistics (mean±SD, median and interquartile range –between 25% and 75% quartiles-) of data were given in Tables. Kolmogorov-Smirnov test was used for normality. Wilcoxon paired signed rank test

was used to compare saline versus L-NAME and saline versus D-NAME applications, because the data distribution was not normal and the variations among measurements were large. In addition, Mann-Whitney U test was used to compare L-NAME and D-NAME groups about the measured variables. In addition, the differences between the proportions of exhibiting aggressive behavior in saline versus L-NAME and saline versus D-NAME applications were evaluated with McNemar test. Fisher exact test was used to show the difference between L-NAME and D-NAME groups about the aggressive animal proportion. If the P value obtained from calculations was smaller than 0.05, the results would be accepted as statistically significant. SPSS (version 11.5) packet program was used in calculations.

3. Results

In this study, a resident-intruder paradigm was used to evaluate maternal aggression towards female intruders in the lactating rats. By using this paradigm, maternal aggression levels were measured on postpartum Day 2 and 3 with applications of saline and 60 mg/kg i.p L-NAME (n=20), respectively. The same protocol was carried out with D-NAME at 60 mg/kg i.p. dose in D-NAME group (n=18).

The proportion of nonaggressive animals which did not exhibit any aggressive behavior during both saline and drug applications was 47% (18/38, n=38). They were excluded from other calculations of this study. Ten out of twenty remained animals belonged to L-NAME group and the other ten belonged to D-NAME group.

The aggressive animal proportion was reduced significantly 70% (7/10, n=10) by L-NAME application (P=0.025), whereas it was reduced only 10% (1/10, n=10) by D-NAME application (P=0.91) compared to previous saline injections which were accepted as control (Fig.1). L-NAME had more significant reducing effect on the aggressive animal proportion than D-NAME (P=0.02). A decrease in the aggression levels was detected in only 1 rat out of

3 which showed aggression in L-NAME group, whereas an increase was observed in the other two.

Results of statistical analysis which compared saline and drug applications are shown in Tables 1 and 2. The latency of first aggressive behavior was significantly increased by L-NAME compared to saline injections (P=0.017). Aggression levels had tendency to reduce by the application of L-NAME in measured parameters such as total number, total duration and the intensity of aggressive behaviors in L-NAME group, however, these effects were not statistically significant (Table 1). On the other hand, D-NAME did not cause any significant change in aggression levels compared to saline injections (Table 2).

In addition, we compared L-NAME group versus D-NAME group on drug effectiveness. We found that L-NAME effect on parameters of maternal aggression was not different than that of D-NAME significantly, except the latency of first aggressive behavior which was significantly longer than D-NAME group (P=0.011).

4. Discussion

One of the most important findings of this study is that the systemic (i.p.) administration of L-NAME, a nonspecific NOS inhibitor, has reducing effects on maternal aggression towards female intruders in the lactating rats while its ineffective enantiomer D-NAME has none. According to our findings, 60 mg/kg i.p. L-NAME significantly delayed the latency of first aggressive behavior. However, it reduced the total duration, total number and the intensity of aggressive behaviors which were not statistically significant. D-NAME was ineffective on all parameters of the aggression test. These results suggest that the decreasing effect of systemically administered L-NAME on maternal aggression might be due to NO production in brain. Our results were similar to previous studies and have provided further

evidence that NO might also be a necessary neuromodulator for expression of maternal aggression.

Another significant finding is the possibility that NO might have a role only on the starting of maternal aggression. Actually we can speculate this possibility because L-NAME application affected significantly only the proportion of aggressive animal (3/10) and the latency to first attack compared to saline injections (Fig. 1). The other parameters of aggression did not change significantly because an increase in the aggression level was observed after L-NAME application in two rats, i.e. once maternal aggression started, it continued even more intensively. Perhaps, NO has different roles in the starting and the continuation of maternal aggression. Further detailed studies are needed to reveal this possibility.

Gammie and Nelson (2001) have used naive Wild-type house mice and found that the aggressive animal proportion was about %57 (n=14) in their study. Another study reported that the proportion of aggressive animal was about 75% in Wistar rats (De Almeida R.M.M. *et al.* 2005). We found in our laboratory that this proportion was 53% in Wistar rats. We excluded animals which were non-aggressive (47%) in both saline and drug applications from all other statistical analysis, because we thought that they did not contribute to the aims of the study which was related to the effects of drugs on maternal aggression. These results showed that researchers have to consider this matter. Pretesting of animals is a useful method to eliminate nonaggressive animals.

In studies conducted so far, only indirect evidences exist about NO production and its levels in brain regions related to behaviors in aggressive and non-aggressive animals (Gammie *et al.* 2000a, Gammie *et al.* 2000b, Nelson *et al.* 1995, Nelson and Trainor 2007). In these studies, NO production was inhibited either by using NOS inhibitors such as 7 Br-Nitroindazole or by using $nNOS^{-/-}$ and $eNOS^{-/-}$ knockout mice, and citrulline-positive cells,

which are indirect indicators of NO production, were counted in various regions of brain. According to the results obtained, in animals which show maternal aggressive behavior the citrulline-positive cell number were found to be significantly higher than that of non-aggressive. There is need to investigate in detail both NO level and the levels of other hormones and modulators which might affect maternal aggressive behavior in aggressive and non-aggressive animals. When different subjects are used in different groups, i.e. between subject designs, three confounded effects might appear. These negative effects are as follows: a) unknown baseline measures b) genetic differences c) environmental differences. Confounded effects cause bias in estimating drug effects. These kinds of experimental designs were sometimes used in studies investigating the role of NO on maternal aggression and this situation may devaluate the results of these studies. We used a paired design to eliminate the negativity mentioned above.

In previous studies using mice and prairie voles, it was reported that NO has an increasing effect on maternal aggression but a reducing effect on male aggression (Gammie *et al.* 2000b, Nelson *et al.* 1995). Therefore, the results of this study are consistent with previous studies and provide further evidence for the point that NO has an increasing role on maternal aggression in other rodent species such as mice and prairie voles. There is only one study about the role of NO in maternal aggression in rats. This study has revealed the fact that application of 250µg L-NAME (the highest dose) into the third ventricle and application of 40µg L-NAME (the highest dose) into the MPOA disrupt maternal aggression (Service and Woodside 2007). In our study which examined the effects of systemic application of L-NAME, similar results were obtained. In our study, reducing effect of L-NAME on maternal aggression may have occurred either via directly inhibiting NO secretion in the related brain regions or indirectly by changing the secretion of other hormone and neurotransmitter

modulated by NO. Further studies are needed to ascertain relations between these hormones and neurotransmitters and NO secretion.

Another difference of our study from previous studies is the sex of the intruder. Maternal aggression is generally considered as defensive behavior but it has been suggested that maternal aggression is a heterogeneous phenomenon ranging from offensive to defensive attack according to the intruder sex (Brain 1990, Oliver and Young 2002). Sexually naive males are particularly subject to intense defensive attack whereas virgin and other female intruders attack offensively. Rats are maximally aggressive in the first lactation week towards female intruders, whereas mice are more aggressive towards male intruders (Brain 1990). Our study has been the first in which female intruders were used and the effects of L-NAME on maternal aggression were similar to studies in which male intruders were used. NO may have similar neural circuits in offensive and defensive type aggression in the lactating rats.

The study had some limitations. The first is the probability of the effects of L-NAME on maternal aggression via changes in blood pressure and brain blood flow. Although, it is known that nonspecific NOS inhibitor L-NAME causes an increase on blood pressure, there is no certain information that hypertension reduces aggression in animals (Huang *et al.* 1995). Furthermore, no reduction in the aggression has been observed by hypertension treatment in hypertensive $eNOS^{-/-}$ knockout male mice (Gammie *et al.* 2000a). Another evidence is that the idea that the effect of L-NAME might be independent of blood pressure is getting similar results in aggression studies with specific nNOS inhibitor 7-NI (Gammie *et al.* 2000b). The second limitation of the study is the possibility that L-NAME may decrease the locomotor activity. However, our previous experiences as well as several reports indicate that NOS inhibitors such as i.p. 60mg/kg L-NAME produce no significant effect on spontaneous locomotor activity of rodents (Celik *et al.* 1999, Johansson *et al.* 1997, Kaputlu and Uzbay 1997, Uzbay and Kayir 2003). Another limitation may be the order effect of test days because

aggression may decrease over time, rather than any effects attributable to the drug. We didn't find any significant order effect of test days in our unpublished study. On the other hand, there are many studies which report that maternal aggression does not decline in the first week after delivery (Haney *et al.* 1989, Mayer 1987, Oliver and Young 2002).

From animal research, the evidence is very strong that there are specific neural substrates in brain subserving different functions in agonistic behavior, and it is more than likely that similar mechanisms are available in the human brain (Oliver and Young 2002). In a study on humans, Rujescu *et al.* showed that some NOS-I haplotypes (C-G-G and G-G) are associated with increased aggression in suicide attempters, some others like C-T-A, T-A and the rs891512 haplotypes of NOS-III are associated with decreased anger-related behavior (Rujescu *et al.* 2008).

In conclusion, our study showed that systemic application of nonspecific NOS inhibitor L-NAME, but not D-NAME, decreases maternal aggression in Wistar rats. This result provides further evidence that NO might be one of the possible mediators on the exhibition of maternal aggression in rats. NO may have a role in offensive maternal behaviors as well as defensive. There might be some confounding effects in maternal aggression studies and therefore a paired study design is more proper to investigate drug effects.

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FIGURE LEGEND

Fig.1. Shows the effect of applying 60mg/kg L-NAME or D-NAME i.p. on maternal aggression in rats. L-NAME application reduced the proportion of mothers exhibiting aggression by 70% than saline application in L-NAME group (P=0.025, n=10). However, the effect of D-NAME was found no statistically significant (P=0.91, n=10). (*P<0.05 statistically significant)

TABLE 1

Table I. Descriptive statistics of variables measured in L-NAME group

Treatment / Measured Variables	**Saline**	**L-NAME**	**P**
	Mean ± SD / Median (IR) †	**Mean ± SD / Median (IR)**	
The latency of first aggressive behavior (sec)	310.50± 140.56/ 282 (183.5–452.5)	887.1 ± 504.44 / 1200 (191.25 – 1200)	**0.017***
The total number of aggressive behavior	4.80 ±3.85 / 3 (2 – 8.5)	4.20 ± 7.09 / 0 (0 –10.5)	0.646
The total duration of aggressive behaviors (sec)	51.30 ± 59.24 / 25 (8.75 – 96.25)	27 ± 45.71 / 0 (0 – 67.50)	0.139
The intensity of aggressive behavior	14.70 ± 13.82 / 11 (3.75 – 20.50)	7.80 ± 14.08 / 0 (0 – 16)	0.139

*P < 0.05 (statistically significant); † IR : interquartile range

TABLE 2

Table II. Descriptive statistics of variables measured in D-NAME group

Treatment / Measured Variables	Saline	D-NAME	P
	Mean ± SD / Median (IR) †	**Mean ± SD / Median (IR)**	
The latency of first aggressive behavior (sec)	252.2± 199.56/ 165.0 (125 – 414)	233.0± 176.05/ 202.5 (102.5 – 345)	0.610
The total number of aggressive behavior	9.70± 5.71/ 10 (4.75 – 12.25)	8.90± 7.47/ 7 (3.0 – 14.75	0.553
The total duration of aggressive behaviors (sec)	53.0± 36.51/ 45 (27.50 – 69.75)	52.30 ± 41.67/ 37.5 (24.5 – 80.5)	0.878
The intensity of aggressive behavior	13.40± 12.71/ 9.50 (4.75 – 17.25)	12.30± 12.99/ 6.5 (3 – 24.25)	0.721

† IR : interquartile range