Monitoring extracellular glutamate in the rat brain by microdialysis and microsensors
van de Zeyden, Miranda

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Chapter 4

The pharmacology of NMDA glutamate receptors \textit{in vivo} studied by a dual probe microdialysis of the ventral tegmental area and nucleus accumbens of the rat brain

Abstract ...................................................................................................................... 78
1. INTRODUCTION ........................................................................................................ 79
2. MATERIALS AND METHODS ....................................................................................... 81
   2.1 Animals .................................................................................................................. 81
   2.2 Materials (drug treatment and doses) ..................................................................... 81
   2.3 Surgery and brain dialysis probes ......................................................................... 81
   2.4 Microdialysis Experiments .................................................................................. 82
   2.5 Chemical assays ................................................................................................. 82
   2.6 Expression of results and statistics ...................................................................... 83
3. RESULTS ..................................................................................................................... 84
   3.1 Basal values ......................................................................................................... 84
   3.2 Determining proper implantation ........................................................................ 84
   3.3 Control experiments: Effect of handling and stress caused by saline injection on the release of dopamine in the NAc .................................................. 85
   3.4 Effect of infusion of different concentrations of NMDA into the VTA on the dialysate content of dopamine in the ipsilateral NAc ........................................... 85
   3.5 Effect of infusion of AP-5, MK801 and ketamine into the VTA on tonic activation of NMDA-receptors in the dialysate content of dopamine in the ipsilateral NAc .................................................................................. 86
   3.6 Effect of infusion of different glycine and D-serine VTA on the dialysate content of dopamine in the ipsilateral NAc ........................................................................... 87
4. DISCUSSION .............................................................................................................. 93
   4.1 Verifying the dual probe implantation .................................................................. 93
   4.2 Tonic regulation of NMDA receptor ..................................................................... 94
   4.3 Effects of stress .................................................................................................... 96
   4.4 Glycine and D-serine .......................................................................................... 96
5. CONCLUSION .......................................................................................................... 98
Abstract
The significance and origin of glutamate sampled by microdialysis is still a matter of debate. An alternative way to study glutamate receptors in vivo is to use a discrete neuronal system on which the presence of NMDA receptors on cellbodies/dendrites is well described. In the present study we have used and evaluated the mesolimbic dopamine system as an in vivo NMDA-receptor model. By infusing NMDA-related compounds into the ventral tegmental area (VTA) and by simultaneously recording dopamine in the ipsilateral nucleus accumbens (NAc) a dual-probe microdialysis system is obtained, which can be used to study properties of the NMDA receptor in the VTA in freely moving animals.

We could demonstrate that the mesolimbic dopamine neurons are tonically excitated by glutamatergic neurons via the NMDA receptor complex. However, the tonus under baseline conditions is low, as only a small non-significant decrease of ±20% was seen when NMDA antagonists were applied. It is concluded that NMDA receptors in the VTA have a significant but modest contribution the tonic activity of the mesolimbic dopamine neurons. Therefore the present model displays a limited sensitivity when a decrease in NMDA-receptor activity needs to be detected.

In contrast increased effect of glutamatergic excitation was clearly detectable as infusions of NMDA increased dopamine levels to about 155% of basal levels in the NAc, when NMDA was infused in the VTA at a concentration of 100 µM. However higher concentrations of NMDA induced a strong behavioural activation. The latter observation limits the usefulness of the method at doses > 100 µM NMDA, as it is difficult to separate pharmacological and stress-induced activation.

Finally, we investigated in the present model whether the NMDA receptor ligands glycine and D-serine have different main points of action. In this study the glycine modulatory site of the NMDA complex in the mesolimbic system seemed to be more susceptible to glycine than D-serine as an increase in dopamine release was observed in the NAc after glycine infusion into the VTA, which was not observed with D-serine.
1. INTRODUCTION

Glutamate is a major excitatory neurotransmitter in the mammalian central nervous system and plays an important role in normal brain functioning as well in pathological conditions ranging from neurotoxicity to neuropsychiatric disorders. Interactions between dopamine and glutamate in the mesocorticolimbic system of the brain are implicated in the regulation of motivated behaviour as well as the pathophysiology of psychiatric disorders such as schizophrenia and drug abuse (Javitt 2007).

The N-methyl-D-aspartate (NMDA) receptor is fundamental to excitatory glutamatergic transmission and critical for normal brain function, including synaptic plasticity and memory formation. Dysfunctions include stroke, alcoholism and epilepsy (Tranquillini and Reggiani 1999; Hoffman 2003; Hoyte et al. 2004). NMDA glutamate receptors are abundant, ubiquitously distributed throughout the CNS. In addition to agonist-binding sites for glutamate, NMDA receptors contain co-agonist sites, called glycine modulatory sites for glycine and D-serine. Importantly, occupation of the glycine modulatory site is required for the glutamate-induced neurotransmission (Johnson and Ascher 1987), indicating that glycine and D-serine facilitate various functions of the NMDA receptor.

To obtain more detailed information about the in vivo properties of brain neurotransmitter receptors, microdialysis studies are often carried out. In the case of the NMDA receptor, microdialysis of glutamate is a possible approach to study this receptor in vivo, however the significance and origin of glutamate in dialysates is still a matter of controversy (Herrera-Marschitz et al. 1996; Timmerman and Westerink 1997; Baker et al. 2002). Glutamate is found in important quantities in neurons as well as in glial cells (astrocytes). Moreover glutamate participates in metabolic routes, which makes it difficult to differentiate between its role as a neurotransmitter and its role in neuronal metabolism (Conti and Weinberg 1999; Watkins 2000; Cakir et al. 2007). The dialysate glutamate levels can arise therefore from synaptic (neuronal) as well as from non-neuronal pools.

An alternative way to study glutamate receptors in vivo is to use a discrete neuronal system on which the presence of NMDA receptors on cellbodies/dendrites is well described (Westerink et al. 1996; Westerink et al. 1997; Mathé et al. 1999). In the present study we have used and evaluated the mesolimbic dopamine system as an in vivo NMDA-receptor model. By infusing NMDA-related compounds into the ventral tegmental area (VTA) and by simultaneously recording dopamine in the ipsilateral nucleus accumbens (NAc) a dual-probe microdialysis system is obtained, which can be used to study properties of the NMDA receptor in the VTA in freely moving animals.
Chapter 4

First the location of the two microdialysis probes was functionally verified by suppressing the dopamine neurons by infusing the GABA-B agonist baclofen into the VTA and recording dopamine in the NAc. Next the optimal concentration for receptor activation of NMDA was determined. To determine the degree of tonic activation of the NMDA-receptor, several receptor blockers (AP-5, MK801, ketamine) were infused into the VTA. Finally the effect of glycine and D-serine on the activity of NMDA-receptors was determined.
2. MATERIALS AND METHODS

2.1 Animals

Male albino rats of a Wistar-derived strain (275-350 g; Harlan, Zeist, The Netherlands) were used for the experiments. The rats were housed individually in plastic cages (35 x 35 x 40 cm) with food and water ad libitum. Animals were kept on a 12 h light schedule (from 7 A.M. until 7 P.M). The experiments were concordant with the declaration of Helsinki and were approved by the Animal Care Committee of the Faculty of Mathematics and Natural Sciences of the University of Groningen, The Netherlands.

2.2 Materials (drug treatment and doses)

The following drugs were used: D,L-baclofen, N-Methyl-D-aspartic acid (NMDA) (purchased from Tocris Neuramin, Essex, England). (±)2-Amino-5-phosphopentanoic acid (AP5), Dizocilpine maleate [(+)-MK-801] (purchased from Research Biochemicals Inc, Natrick, USA). Serine, L-Cystine, Glycine and Tetrodotoxin (TTX) (purchased from Sigma-Aldrich B.V., Zwijndrecht, the Netherlands). The drugs were first dissolved in water at a concentration of 10 µM and further diluted with Ringer. Drugs dissolved in Ringer were infused via retrograde microdialysis into the VTA. The composition of the Ringer’s solution was (in mM) NaCl 147.0, KCl 3.0, CaCl₂ 1.2 and MgCl₂ 1.0. One of the conditions of the dual-probe technique is that infused concentrations of drugs should be sufficiently high to block or stimulate the appropriate receptors in the infused area (here aimed at the VTA). Infused doses were based on earlier studies in related experiments. To induce stress rats were handled. When rats were handled, the animals were picked up from the home cage and handled for 15 min, so that they were not allowed to escape.

2.3 Surgery and brain dialysis probes

Microdialysis was performed with two I-shaped microdialysis probes constructed with a regenerated cellulose or Hospal AN67HF:polyacrylnitrile dialysis fibre membrane (Brainlink, Groningen, The Netherlands), with a i.d 220 µm and o.d 310 µm. One probe (exposed length 1.5 mm) was implanted into the VTA, and the second probe (exposed length 1.5 mm) was implanted into the ipsilateral NAc. The probe implanted in the VTA was used to deliver drugs; the probe implanted in the ipsilateral NAc was used to record extracellular dopamine. Coordinates of the implantation were as follows: A/P 2.2, L/M -1.6 and V/D -7.2 (NAc); A/P -5.0, L/M +0.9, and V/D -8.0 (VTA), from bregma point and dura. The dialysis probes were stereotactically implanted in the animal brain under the following conditions: isoflurane 2%, O₂, 600 ml/min and local bupivacainehydrochloride monohydraat (Marcaine 0.5% adrenaline) anaesthesia. The microdialysis probes were permanently fixed to the skull using stainless steel screws and methylacrylic cement.
Rats were placed in individual perspex cages (35x35x40 cm) where they had free access to food and water. Animals were allowed to recover 24 hrs before microdialysis experiments commenced.

2.4 Microdialysis Experiments

Microdialysis experiments were carried out 24-72 hr after implantation of the probes. In all experiments the dialysis probes were perfused with a Ringer solution at a flow rate of 1.5 µl/min with aid of a pump (Technical and Scientific Equipment (TSE), Bad Homburg, Germany). All pharmacological agents used for infusion were prepared in this Ringer solution as described. All inlet and outlet tubing was from flexible PEEK (I.D. 0.15 mm; Watson-Marlow).

An on-line approach was used to determine dopamine in the NAc where the dialysate was on-line collected into an HPLC injection loop and automatically injected every 15 min. After 4 basal samples were collected, drug infusion were performed for the duration of the experiment, followed by reperfusion of the Ringer solution.

Implantation of the probes was first evaluated functionally as described below. The experiments were completed with infusion of 50 µM baclofen or 1 µmol/l TTX into the VTA probe, and the response in the NAc was determined. A decrease in extracellular dopamine in the NAc to at least 40% of baseline was considered as a measure for correct implantation. When the experiment was terminated, the animal was anaesthetized with isofluoran and then euthanized with 1.0 mg/kg sodiumpentobarbital. The brain was fixed with 4% paraformaldehyde. Coronal sections (40 µm thick) were made, and dialysis probes placement verified according to the atlas of (Paxinos and Watson 1986).

2.5 Chemical assays

Dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC) and 5-hydroxyindoleacetic acid (HIAA) was quantified by HPLC coupled with electrochemical detection. A Shimadzu (LC-10AD) pump was used in conjunction with an electrochemical detector (ESA Coulochem II), with a Coulochem 5011 detector cell, potential first cell: +500 mV; potential second cell: -250 mV). A reverse-phase column (150 x 4.7 mm²; Supelco LC18, Bellefonte, PA) was used. The mobile phase consisted out of mixture of 0.05 M sodium acetate, 1 M octanesulfonic acid, 0.5 mM Na₂-EDTA, and 130 ml/L methanol adjusted to pH 4.2 with acetic acid, at a flow of 1.0 mL/min. The detection limit of the assay was ±3 fmol/sample (on-column).
2.6 Expression of results and statistics

All values given are expressed as percent of the baseline. The average concentration of four consecutive base-line samples (less than 10% variation) was defined as 100%. Statistical analysis (SPSS14.0 for windows) was performed using one-way analysis of variance for repeated measures followed by the post-hoc Dunnett’s multiple comparison testing. The level of significance was set at p<0.05.
3. RESULTS

3.1 Basal values

Baseline levels in the NAc samples were determined as (± S.E.M) for DA 3.38 ± 0.3 fmol/min and for DOPAC and HIAA (± S.E.M) 0.91 ± 0.08 and 0.22 ± 0.02 pmol/min (n=55).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mechanism of action</th>
<th>Dose µM</th>
<th>Basal DA fmol/sample</th>
<th>Basal DOPAC pmol/sample</th>
<th>Basal HIAA pmol/sample</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baclofen</td>
<td>GABA&lt;sub&gt;B&lt;/sub&gt; agonist</td>
<td>50</td>
<td>5.52 ± 0.94</td>
<td>0.62 ± 0.05</td>
<td>0.22 ± 0.04</td>
<td>5</td>
</tr>
<tr>
<td>NMRA</td>
<td>NMRA agonist</td>
<td>200</td>
<td>3.58 ± 1.14</td>
<td>0.88 ± 0.10</td>
<td>0.24 ± 0.05</td>
<td>5</td>
</tr>
<tr>
<td>Handling</td>
<td>stress</td>
<td>15 min</td>
<td>3.72 ± 1.54</td>
<td>1.07 ± 0.16</td>
<td>0.24 ± 0.03</td>
<td>4</td>
</tr>
<tr>
<td>Saline IP</td>
<td>stress</td>
<td>1 ml/kg</td>
<td>4.89 ± 1.94</td>
<td>1.16 ± 0.177</td>
<td>0.24 ± 0.01</td>
<td>4</td>
</tr>
<tr>
<td>NMRA</td>
<td>NMRA agonist</td>
<td>10</td>
<td>2.99 ± 0.96</td>
<td>0.69 ± 0.22</td>
<td>0.21± 0.05</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
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<td>200</td>
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<td></td>
<td></td>
<td>300</td>
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<tr>
<td>AP-5</td>
<td>competitive NMRA antagonist</td>
<td>500</td>
<td>3.55 ± 1.07</td>
<td>0.80 ± 0.19</td>
<td>0.22 ± 0.05</td>
<td>4</td>
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<tr>
<td>MK-801</td>
<td>noncompetitive NMRA antagonist</td>
<td>1000</td>
<td>4.42 ± 2.17</td>
<td>1.18 ± 0.37</td>
<td>0.22 ± 0.03</td>
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<tr>
<td>Glycine</td>
<td>NMRA agonist</td>
<td>10</td>
<td>2.44 ± 0.50</td>
<td>1.28 ± 0.25</td>
<td>0.24 ± 0.03</td>
<td>4</td>
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<td></td>
<td></td>
<td>100</td>
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<td></td>
<td></td>
<td>1000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serine IP</td>
<td>NMRA agonist</td>
<td>300</td>
<td>3.74 ± 0.74</td>
<td>1.30 ± 0.48</td>
<td>0.39 ± 0.11</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Selective glycine&lt;sub&gt;B&lt;/sub&gt; agonist</td>
<td>1000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serine</td>
<td>NMRA agonist</td>
<td>10</td>
<td>1.89 ± 0.90</td>
<td>0.75 ± 0.16</td>
<td>0.18 ± 0.03</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Selective glycine&lt;sub&gt;B&lt;/sub&gt; agonist</td>
<td>1000</td>
<td></td>
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</table>

3.2 Determining proper implantation

Effect of infusion of Baclofen into the VTA on the dialysate content of dopamine in the ipsilateral NAc

The GABA<sub>B</sub> agonist baclofen was infused continuous into the VTA at a concentration of 50 µM. This GABA<sub>B</sub> agonist caused a decrease of extracellular dopamine in the ipsilateral NAc to ±16% of baseline. This decrease was statistically significant 15 min after start of infusion (F<sub>1,30</sub>=62.358; p<0.000, n=5) (Figure 1). Baclofen at a concentration of 50 µM is a significant concentration (F<sub>1,43</sub>= 86.716 p<0.000).
Effect of infusion of NMDA into the VTA on the dialysate content of dopamine in the ipsilateral NAc

NMDA was infused into the VTA at a concentration of 200 µM. Because of the strong behavioural activation, the NMDA infusion was restricted to 30 min. The strong behavioural activation, including hyperlocomotion, turning behaviour, rearing and grooming started after 15 min of infusion and lasted for about 15 min after infusion was stopped, after which the animals returned to their usual resting state. The extracellular dopamine in the ipsilateral NAc increased to ±206% of baseline (Figure 2). The increase was statistically significant 15 min after the start of the infusion (F_{15,90}=6.140; p<0.000, n=5). NMDA at a concentration of 200 µM is a significant concentration (F_{1,79} = 6.997 p=0.010).

Consistent with the fact that dopamine neurons originate in the A10 region of the VTA in the brainstem and innervate the NAc (often referred to as the ventral striatum), the effect of 50 µM baclofen and 200 µM NMDA was used as a functional test for proper implantation of the probe. The baclofen and NMDA effect demonstrates that the two probes are properly implanted with respect to the mesolimbic partway.

3.3 Control experiments: Effect of handling and stress caused by saline injection on the release of dopamine in the NAc

Effect of handling on the release of dopamine in the nucleus accumbens

Gentle handling during 15 min induced an increase in extracellular dopamine in the NAc to ±127% of baseline. The effect reached its maximum 30 min after the start of the handling and lasted about 75 min. This handling induced increase was not statistically significant in time (F_{13,39}=2.015; p=0.230, n=4) (Figure 3).

Effect of saline injection on the release of dopamine in the nucleus accumbens

Intra peritoneal injection of saline did not have any influence on the extracellular dopamine levels in the NAc in time (F_{11,33}=0.602; p=0.814, n=4). (Figure 4).

3.4 Effect of infusion of different concentrations of NMDA into the VTA on the dialysate content of dopamine in the ipsilateral NAc

N-methyl-D-aspartic acid was perfused during 30 min through the microdialysis probe in the VTA at three different concentrations to evaluate a possible dose-response relationship (Figure 5 and 6). Infusion of 10, 50 and 100 µM NMDA into the VTA did not induce any significant change in the NAc dopamine basal value over time. (F_{21,84}=1.443;
Chapter 4

p=0.122, n=5). A trend to increase to ±131% when 100 µM NMDA is applied was observed. A significant difference was therefore found for the concentration 100 µM NMDA compared to the baseline values (F\textsubscript{3,109}=5.628; p=0.001).

Infusion of 100, 200 and 300 µM NMDA into the VTA induce a significant change in the NAc dopamine basal value over time (F\textsubscript{21,168}=3.036; p<0.000, n=9) (Figure 6). For 100, 200 and 300 µM NMDA the average increase was ±139, ±148, and ±156% respectively. Each concentration was found to be significant to the baseline values in the test for dose dependency (F\textsubscript{3,194}=7.706; p<0.000)

3.5 Effect of infusion of AP-5, MK801 and ketamine into the VTA on tonic activation of NMDA-receptors in the dialysate content of dopamine in the ipsilateral NAc

Effect of infusion of AP5 into the VTA on the dialysate content of dopamine in the ipsilateral NAc

During infusion of the competitive NMDA-antagonist AP5, at a concentration of 500 µM into the VTA, the extracellular dopamine in the ipsilateral NAc decreased to about ±84% of baseline (Figure 7). This decrease was statistically not significant (F\textsubscript{14,42}=1.670; p=0.100, n=4). Sequential co-infusion of 500 µM AP5 with 200 µM NMDA did not significantly increase the dialysate dopamine levels, indicating that AP-5 fully inhibited NMDA. AP-5 also fully removed the behavioural effects induced by NDMA. The concentration of AP5 co-administered with NMDA was found to be significant different compared to AP5 alone (F\textsubscript{2,59}= 6.355; p=0.003)

Effect of infusion of MK801 into the VTA on the dialysate content of dopamine in the ipsilateral NAc

During intrategmental infusion of 1 mM (+)-MK-801, extracellular dopamine in the ipsilateral NAc decreased to about ±83% of baseline (Figure 8). This decrease did not reach statistical significance. Sequential intrategmental infusion of the noncompetitive NMDA antagonists (+)-MK-801 with NMDA suppressed the increase induced by 200 µM NMDA (F\textsubscript{16,48}=1.478; p=0.148, n=4). The hyperlocomotion which was observed when administration 200 µM NMDA was completely blocked by co-infusion with (+) MK-801. Statistical significance were found in concentration; 1 mM (+)-MK-801 compared to the basal samples (p=0.003) and 1 mM (+)-MK-801 combined with 200 µM NMDA compared to 1 mM (+)-MK-801 alone (p=0.035) (F\textsubscript{2,67}= 2.898; p=0.062).
3.6 Effect of infusion of different glycine and D-serine VTA on the dialysate content of dopamine in the ipsilateral NAc

Effect of infusion of glycine into the VTA on the dialysate content of dopamine in the ipsilateral NAc

Infusion of glycine for 90 minutes in the VTA induced a significant increase to ±170% of dopamine basal values in the ipsilateral NAc ($F_{21,63}=6.659; p<0.000, n=4$). More specific infusion of 10, 100 µM and 1 mM glycine caused an increase of ±109%, ±126% and ±147% respectively (Figure 9). Each concentration was found to be significant to the baseline values in the test for dose dependency ($F_{3,87}= 22.920; p<0.000$).

Effect of infusion of D-serine into the VTA on the dialysate content of dopamine in the ipsilateral NAc

The reverse dialysis of the D-serine in the VTA produced no significant effect in dopamine release in the NAc over time ($F_{27,54}=0.325; p=0.999, n=4$) (Figure 10).
Figure 1: Effect of baclofen (50 µM), infused by retrograde dialysis into the VTA, on extracellular levels of dopamine in the ipsilateral NAc. The horizontal bar represents the period of infusion of baclofen. Data are given as percentage of basal values ± SEM (n=5) * indicates post-hoc significance (p <0.05) versus baseline.

Figure 2: Effect of NMDA (200 µM), infused by retrograde dialysis into the VTA, on extracellular levels of dopamine in the ipsilateral NAc. The horizontal bar represents the period of infusion of NMDA. Data are given as percentage of basal values ± SEM (n=5) * # and ' indicates post-hoc significance (p <0.05) versus baseline.
Figure 3: Effect of handling on extracellular levels of dopamine in the NAc. The horizontal bar represents the period of handling. Data are given as percentage of basal values ± SEM (n=4).

Figure 4: Effect of saline injection IP on extracellular levels of dopamine in the NAc. Data are given as percentage of basal values ± SEM (n=4).
Figure 5: Effect of NMDA (10, 50 and 100 µM), infused by retrograde dialysis into the VTA, on extracellular levels of dopamine in the ipsilateral NAc. The horizontal bars represent the period of infusion of NMDA. Data are given as percentage of basal values ± SEM (n=5). # indicates post-hoc significance (p<0.05) versus baseline.

Figure 6: Effect of NMDA (100, 200 and 300 µM), infused by retrograde dialysis into the VTA, on extracellular levels of dopamine in the ipsilateral NAc. The horizontal bars represent the period of infusion of NMDA. Data are given as percentage of basal values ± SEM (n=9). * and # indicates post-hoc significance (p<0.05) versus baselines.
The pharmacology of NMDA glutamate receptors in vivo studied by a dual probe microdialysis of the VTA and NAc of the rat brain

Figure 7: Effect of AP5 (500 µM) sequentially followed by co-administration of AP5 with NDMA (500 µM AP5 and 200µM NMDA), infused by retrograde dialysis into the VTA, on extracellular levels of dopamine in the ipsilateral NAc. The horizontal bar represents the period of infusion of AP5 and AP5 with NMDA. The dotted line indicates the graph of 200 µM NMDA infusion alone. Data are given as percentage of basal values ± SEM (n=4).

Figure 8: Effect of MK801 (1 mM) sequentially followed by co-administration of MK801 with NDMA (1 mM MK801 and 200 µM NMDA), infused by retrograde dialysis into the VTA, on extracellular levels of dopamine in the ipsilateral NAc. The horizontal bar represents the period of infusion of MK801 and MK801 with NMDA. The dotted line indicates the graph of 200 µM NMDA infusion alone. Data are given as percentage of basal values ± SEM (n=4).
Figure 9: Effect of glycine (10, 100 µM and 1 mM) infused by retrograde dialysis into the VTA, on extracellular levels of dopamine in the ipsilateral NAc. The horizontal bar represents the period of infusion of glycine. Data are given as percentage of basal values ± SEM (n=4) * indicates post-hoc significance (p <0.05) versus baseline.

Figure 10: Effect of D-serine (10 µM, 100 µM, 1 mM and 10 mM), infused by retrograde dialysis into the VTA, on extracellular levels of dopamine in the ipsilateral NAc. The horizontal bar represents the period of infusion of serine. Data are given as percentage of basal values ± SEM (n=4).
4. DISCUSSION

In the present study we have elaborated/worked out an *in vivo* NMDA receptor model that can be applied to conscious animals. The model is based on glutamate NMDA receptors located on somatodendritic dopamine neurons in the mesolimbic pathway. We have utilized a dual probe microdialysis technique in freely moving rats where we manipulated the glutamatergic afferent input to the dopamine neurons by infusing NMDA receptor compounds via a microdialysis probe (probe 1) in the VTA, while concurrently recording the effect of this modulation on the output of the dopamine neurons as determined by the release of dopamine in the ipsilateral NAc (probe 2). The method is an elaboration of our earlier studies (Westerink *et al.* 1996; Westerink *et al.* 1997). Dual probe analysis in these brain areas looking at the NMDA receptor have been performed in related studies (Wang *et al.* 1994; Westerink *et al.* 1996; Karreman *et al.* 1996; Westerink *et al.* 1997; Schilström *et al.* 1998; Mathé *et al.* 1999; Kretschmer 1999; Ericson *et al.* 2003).

4.1 Verifying the dual probe implantation

The two microdialysis probes locations were verified by inhibition of the mesolimbic pathway through infusion of the GABA$_B_3$ agonist baclofen and by activation of the pathway by NMDA into the VTA, simultaneously dopamine was recorded in the ipsilateral NAc.

GABA$_B_3$ receptors are present on somatodendritic sites of mesolimbic dopamine neurons (Klitennick *et al.* 1992), therefore infusion of baclofen into the VTA caused a pronounced decrease in extracellular dopamine in the ipsilateral NAc, confirming the inhibition of the mesolimbic pathway. This decrease is in accordance with earlier studies (Westerink *et al.* 1996; Westerink *et al.* 1997).

NMDA receptors are present on somatodendritic sites of mesolimbic dopamine neurons. Infusion of NMDA in the VTA induced a pronounced increase in extracellular dopamine in the ipsilateral accumbens. These results are in accordance with earlier studies (Suaud-Chagny *et al.* 1992; Wang *et al.* 1994; Westerink *et al.* 1996; Karreman *et al.* 1996; Kretschmer 1999).

Apparently the infused concentrations of baclofen and NMDA were sufficiently high enough throughout the VTA to reach the relevant receptors and induce pronounced
changes in the release of dopamine in the ipsilateral accumbens. Therefore it is concluded that the VTA and NAc probes were properly located.

In order to determine the optimal concentrations to activate the NMDA receptor, NMDA was infused at increasing concentrations, whereas dopamine was recorded in the ipsilateral NAc. NMDA was found to be effective in the micromolar range 10-300 µM. The progressive increase did not reach equilibrium during the time of infusion. Equally, behavioural activity increased with increasing concentrations of NMDA. A concentration of 300 µM NMDA induced a strong hyperactive behaviour, to such an extent that a higher dosage of NMDA was considered undesirable.

4.2 Tonic regulation of NMDA receptor
To establish the degree of tonic activation in the in vivo NMDA receptor model different NMDA receptor blockers were infused into the VTA. Both D-(-)-2-Amino-5-phosphonopentanoic acid (AP5) a potent and selective competitive NMDA-type glutamate receptor antagonist and Dizoclipine (MK-801) a potent and selective non-competitive NMDA receptor antagonist decreased dopamine release in the mesolimbic pathway, although these effects did not reach the level of statistical significance. However stimulation of the dopamine pathway by NMDA was largely suppressed during co-infusion of AP5 or MK-801 into the VTA, confirming that the NMDA-stimulated release of dopamine neurons was mediated by NMDA receptors.

The result obtained after AP5 infusion is consistent with data from previous microdialysis studies. For example (Kretschmer 1999) found a decrease of 76% and 58% of basal dopamine levels in the NAc after infusion of 100 and 200 µM AP5 respectively in the VTA, whereas (Karreman et al. 1996) found a decrease of ±75% of basal dopamine levels in the NAc shell after infusion of a higher concentration (500 µM) AP5 in the VTA. (Westerink et al. 1996;Westerink et al. 1997) infused the competitive NMDA antagonist CPP into the VTA at a concentration of 30 and 100 µM, which did not modify extracellular dopamine in the ipsilateral NAc, although CPP suppressed the increase induced by 15 min infusion of 1 mM NMDA. Electrophysiological data confirm these results, showing that local infusion of competitive NMDA receptor antagonists decrease VTA dopamine neuronal activity by reducing and changing the firing rate and burst firing patterns of midbrain neurons (Chergui et al. 1993). Therefore it can be presumed that a competitive NMDA antagonist will decrease dopamine levels no more than 20% of the basal values in the mesolimbic system.
The pharmacology of NMDA glutamate receptors in vivo studied by a dual probe microdialysis of the VTA and NAc of the rat brain

Similarly the MK-801-induced decrease in dopamine levels, yet not significant in the NAc in the present study is consistent with two other dual-probe microdialysis studies. (Kretschmer 1999) found that MK-801 induced no immediate effect but a decrease response after 220 minutes, following exposure to 1 and 10 µM MK-801. (Westerink et al. 1996) reported a similar observation as 1 mM MK-801 caused a tendency to decrease dopamine levels in the ipsilateral NAc, however this decrease was not significant. Furthermore co-infusion of MK-801 suppressed the increase induced by NMDA (Westerink et al. 1996). Contrary to these results another dual-probe microdialysis study performed by (Ericson et al. 2003) found that MK801 (100 µM) induced a 125% increase in dopamine in the NAc, indicating a bell shape dose response. In another study (Wang et al. 1994) infused the non competitive NMDA antagonist phencyclidine (100 µM PCP) in the VTA which had no effect on the basal extracellular dopamine concentration in the NAc, but did abolish the 100 µM NMDA induced increase.

Where reports have consistently shown that MK-801 has a greater affinity for NMDA receptors than ketamine (Sun and Wessinger 2004), ketamine has become a favourable drug alternative. Ketamine is described as a phencyclidine hydrochloride derivative, dissociative anaesthetic and a non-competitive antagonist of the glutamate NMDA receptor (Krystal et al. 1994). In a pilot study, 0.1-1 mM ketamine perfused in the VTA had no effect on basal dopamine levels in the ipsilateral NAc. (data not shown). There has to our knowledge been, no reports on perfusion administration experiments with ketamine using microdialysis so far. This is rather surprising knowing that systemic application of ketamine increase extracellular dopamine in the striatum (STR), PFC, VTA and NAc (Irifune et al. 1998;Cartmell et al. 1999;Masuzawa et al. 2003).

Results in the present study support that the somatodendritic dopamine neurons in the VTA of the mesolimbic pathway were susceptible to NMDA modulation, as dopamine release in the NAc was affected by NMDA agonists and (to a limited extend) most of the antagonists. The increase of dopamine in the NAc after local VTA administration of NMDA was concentration dependent. These observations confirm earlier studies (Suaud-Chagny et al. 1992;Westerink et al. 1996;Karreman et al. 1996).

The weakness of the NMDA receptor model in the mesolimbic pathway is evident when looking at the small tonic modulation of the NMDA receptors on the VTA dopamine neurons during control conditions. A tonic excitation of 20% was observed, indicating a narrow range of induced change after drug application, making it difficult to draw direct conclusions. The decrease of dopamine caused by the NMDA receptor antagonists was
in line with previous dual-probe microdialysis studies where a 10-20% decrease was found (Westerink et al. 1996; Karreman et al. 1996). It can therefore be concluded that, NMDA receptors in the VTA have a noteworthy but modest contribution to the tonic inhibition/activity of the mesolimbic dopamine neurons. These results further support the fact that the mesocortical system (VTA→PFC) displays a greater tonic sensitivity than the mesolimbic system (VTA→NAc) (Westerink et al. 1998).

4.3 Effects of stress

Various authors have shown that conditions of stress ranging from gentle handling, tail pinch or immobilisation, increases dopaminergic activity in mesolimbic-mesocortical dopamine neuronal pathway. This effect is most pronounced in the mesocortical dopamine neuronal pathway (Enrico et al. 1998). To determine whether experiment related procedures such as handling and injecting would influence data obtained, control experiments were performed. In this study handling and the stress of a saline injection caused a slight increase (±130% and 110% respectively of baseline) in extracellular dopamine in the NAc which did not reach the level of statistical significance.

We realize that infusion of high concentrations of NMDA into the VTA induces turning and locomotor behaviour which may lead to a stressful condition, which might by itself lead to increase of dopamine release. Therefore, we used a concentration of 100 µM NMDA in further experiments, given that this concentration was virtually without effect on behaviour. NMDA at higher concentrations can be used in experiments which are performed under anaesthesia, but we especially wanted to prevent any influence of anaesthetic conditions.

4.4 Glycine and D-serine

Finally we applied the dual-probe model by investigating the effects of glycine and D-serine on NMDA receptor activity. It has been described that the NMDA receptors contain a primary agonist binding site for glutamate and an additional modulatory site for endogenous monocarboxylic amino acid glycine and D-serine (Javitt 2004). Currently the glycine modulatory site on the NMDA receptors, presents an attractive therapeutic target site. It has been reported that the exogenous administration of the glycine site agonists, glycine and D-serine can stimulate NMDA receptors without inducing the toxic effects that are seen after direct agonists such as NMDA and glutamate (Coyle and Tsai 2004).
**Glycine:** Local VTA infusion of glycine produced a significant concentration-dependent increase (±170% of baseline) in extracellular dopamine in the ipsilateral NAc. Since occupation of the glycine modulatory site is required for the glutamate-induced neurotransmission (Johnson and Ascher 1987), increased glycine concentrations can only activate the NMDA complex if there is already glutamate available in the vicinity of the synapse. The present results indicate that there was sufficient glutamate available at the NMDA receptor complex. It can also be concluded that addition of glycine might be useful to further stimulate NMDA receptors.

**D-serine:** In the past decades it has become clear that D-serine should be considered as an important co-transmitter, since on the NMDA receptor (1) D-serine is found in astrocytes that ensheath NMDA-receptor-bearing neurons. (2) The distribution of D-serine is homogenous to the distribution of NMDA receptors where in contrast, glycine distribution does not correspond to NMDA receptor distribution except for some regions where D-serine is also present (Schell et al. 1995). (3) The fact that D-serine is released from astrocytes upon activation of their glutamatergic receptors strongly suggests that glutamate release from the nerve terminal triggers glial D-serine efflux, which in turn modulates the NMDA receptors at postsynaptic sites (Martineau et al. 2006).

D-serine however did not produce any effect on dopamine release in the NAc after infusion in the VTA, while systemic administration of D-serine slightly increased dopamine release in the NAc (±120% of baseline). However, the latter rise can also explained as an administration artefact, as observed for saline (see Fig. 4).

Glycine and D-serine have shown respectively to induce few or no response in the NAc, whereas NMDA induced a clear response. The activation of the glycine modulatory site of the NMDA receptor in the mesolimbic model indicates that the model detects differences between glycine and D-serine. However, it is emphasized that the present model may lack sufficient sensitivity to quantify these differences. It also has to be taken into account that modulation of the NMDA-receptor complex by glycine and D-serine is rather complex. Where these two compounds have similar excitatory effects on NMDA receptors containing NR2 subunits they have in fact opposite effects on NMDA receptors containing NR3 subunits. Glycine serves to activate NR3 containing receptors and D-serine to inhibit them (Chatterton et al. 2002), which confirms that these two compound have intrinsic different modes of action on the NMDA receptor complex in the mesolimbic model.
5. CONCLUSION
Mesolimbic dopamine neurons are tonically excitated by glutamatergic neurons via the NMDA receptor complex. However, the tonus under baseline conditions is low, as only a small non-significant decrease of ±20% was seen when NMDA antagonists were applied. From this minor decrease (which is difficult to quantify with the microdialysis method), it can be concluded that NMDA receptors in the VTA have a significant but modest contribution to the tonic activity of the mesolimbic dopamine neurons. Therefore the present model displays a limited sensitivity when a decrease in NMDA-receptor activity needs to be detected.

In contrast increased effect of glutamatergic excitation was clearly detectable as infusions of NMDA increased dopamine levels to about 155% of basal levels in the NAc when NMDA was infused in the VTA at a concentration of 100 µM. However higher concentrations of NMDA induced a strong behavioural activation. The latter observation limits the usefulness of the method at doses > 100 µM NMDA, as at these concentrations the pharmacological and stress-induced activation are difficult to separate.

Finally, we investigated in the present model whether the NMDA receptor ligands glycine and D-serine have different main points of action. In this study the glycine modulatory site of the NMDA complex in the mesolimbic system seemed to be more susceptible to glycine than D-serine as an increase in dopamine release was observed in the NAc after glycine infusion into the VTA, which was not observed with D-serine.