Good vibrations: Rhythms and plasticity in neural correlates of value in rat orbitofrontal cortex
van Wingerden, E.J.M.

Citation for published version (APA):
CHAPTER 4

NMDA RECEPTORS CONTROL CUE-OUTCOME SELECTIVITY AND PLASTICITY OF ORBITOFRONTAL FIRING PATTERNS DURING ASSOCIATIVE STIMULUS-REWARD LEARNING

M. van Wingerden*1,2, M. Vinck*1, V. Tijms3, I.R.S. Ferreira4, A.J. Jonker5, C.M.A. Pennartz1,3
1Center for Neuroscience, University of Amsterdam, Netherlands
2Institute of Experimental Psychology, Heinrich-Heine University Düsseldorf, Germany
3Cognitive Science Center Amsterdam, Netherlands
4Max Planck Institute of Neurobiology, Munich, Germany
5Department of Anatomy & Neurosciences, Free University Medical Center, Amsterdam, Netherlands.
*These authors contributed equally to this work.

Published as:
Chapter 4. NMDA-R IN OFC PLASTICITY

ABSTRACT
Neural activity in orbitofrontal cortex has been linked to flexible representations of stimulus-outcome associations. Such value representations are known to emerge with learning, but the neural mechanisms supporting this phenomenon are not well understood. Here, we provide evidence for a causal role for NMDA receptors (NMDARs) in mediating spike pattern discriminability, neural plasticity and rhythmic synchronization in relation to evaluative stimulus processing and decision-making. Using tetrodes, single-unit spike trains and local field potentials were recorded during local, unilateral perfusion of an NMDAR blocker in rat OFC. In the absence of behavioral effects, NMDAR blockade severely hampered outcome-selective spike pattern formation to olfactory cues, relative to control perfusions. Moreover, NMDAR blockade shifted local rhythmic synchronization to higher frequencies and degraded its linkage to stimulus-outcome selective coding. These results demonstrate the importance of NMDARs for cue-outcome associative coding in OFC during learning and illustrate how NMDAR blockade disrupts network dynamics.
INTRODUCTION

An essential component of decision-making is the retrieval of values associated with stimuli and utilization of this information to select responses. Value is the net payoff, or outcome, that is predicted to occur in the future given a stimulus or state. Recent studies have shed light on the neuronal correlates of value representations in the brain and how stimulus-outcome associations are updated when task contingencies are changed (e.g. Platt and Glimcher, 1999; Sugrue et al., 2005; Padoa-Schioppa and Assad, 2006). Across several species, the OFC has been consistently implicated in coding and utilizing such representations during decision-making. Stimuli elicit responses in orbitofrontal neurons that are sensitive to future outcome (Hikosaka and Watanabe, 2000; Tremblay and Schultz, 2000a, 2000b; Wallis and Miller, 2003; Padoa-Schioppa and Assad, 2006; van Duuren et al., 2008; Schoenbaum et al., 2009). Complementary lesion studies suggested a causal role of OFC in the updating of stimulus values and the assignment of credit to behavioral choices associated with positive or negative outcome (Rolls et al., 1994; Baxter et al., 2000; Schoenbaum et al., 2002, 2009; Bohn et al., 2003a; Walton et al., 2010). However, the neural mechanisms mediating these OFC functions are largely unknown. Because electrophysiological studies provide correlative data, it has also remained unknown whether neural representations of value depend on mechanisms within orbitofrontal cortex itself.

A promising starting point to investigate these mechanisms is the N-methyl-D-aspartate receptor (NMDAR). This is motivated by the glutamatergic nature of fast excitatory connections in OFC, including its thalamic and cortical afferents as well as intrinsic connections between its pyramidal cells (Wang, 1999; Seamans et al., 2003; Hoover and Vertes, 2011). NMDARs play a key role in synaptic plasticity, including both long-term potentiation and depression (Selig et al., 1995; Lee et al., 1998; Malenka and Nicoll, 1999). The role of NMDARs in mediating learning-related changes in neural excitability in vivo has been primarily studied in amygdala in relation to fear conditioning (Li et al., 1995; Goosens and Maren, 2004), and in hippocampus in relation to spatial memory (Morris et al., 1986; Kentros et al., 1998; Ekstrom et al., 2001; McHugh et al., 2007), but not in the context of associative stimulus-reward learning as exemplified by OFC neurons.

Schoenbaum et al. (1998, 1999) showed that, during learning, OFC
neurons come to fire differentially to stimuli associated with distinct outcomes, but it is unknown whether this selectivity arises from local OFC mechanisms and depends on NMDAR activity. Apart from a hypothesized role in long-term plasticity of OFC firing patterns, NMDARs may contribute acutely to OFC information processing: under depolarized membrane voltages they contribute slow EPSP components to synaptic responses (Herron et al., 1986), and these may help solve e.g. pattern discrimination and working-memory problems (Wang, 1999; McHugh et al., 2007). By the same token, NMDARs may contribute to spike timing relative to the phase of oscillatory local field potentials (LFPs), as hypothesized for hippocampus (Jensen and Lisman, 1996; Buzsáki, 2002). If NMDARs modulate the strength of spike-LFP phase-locking, they would be in a key position to affect the efficacy by which OFC output excites target areas (such as striatum and basolateral amygdala; Pennartz et al., 2011) and to regulate downstream synaptic modifications by spike-timing dependent plasticity (Bi and Poo, 1998; Cassenaer and Laurent, 2007).

We applied the novel technique of reverse microdialysis in combination with multi-tetrode recordings to study how NMDAR blockade affects discriminatory firing patterns and rhythmic mass activity in the OFC of rats learning stimulus-reward associations and reversals in an odor discrimination task (Schoenbaum et al., 1998; van Duuren et al., 2007a). We show that NMDARs affect discriminatory coding of OFC neurons especially during stimulus presentation and decision-making and shape plasticity of discriminatory firing across learning trials. NMDAR blockade leads to hypersynchronous phase-locking in the theta, beta and high-frequency bands, and destroys the functional relationship between theta-band phase-locking and discriminative power by virtue of firing rate. Unilateral blockade of NMDA-receptors does not affect behavioral performance during task acquisition, but hampers changes in reaction time after reversal of task contingencies.

EXPERIMENTAL PROCEDURES

Subjects
The general behavioral methods of this experiment have been reported elsewhere in detail (van Wingerden et al., 2010a, 2010b). Data was collected from four male Wistar rats. Animals were communally housed in standard
cages under a reversed day/night cycle (lights off: 7:00 AM, lights on: 7:00 PM), handled and subjected to behavioral training daily at fixed times. During behavioral training and the main experiment, animals were food restricted to maintain their body weight at 90% of free-fed animals. Two days before surgery and during the subsequent post-surgery recovery week, food was available ad libitum. After surgery, animals were housed individually in transparent cages (40 x 40 x 40 cm). All experiments were conducted according to the National Guidelines on Animal Experiments and with approval of the Animal Experimentation Committee of the University of Amsterdam.

Apparatus
The animals were trained on an operant task in a custom-made operant chamber. Both its odor port and fluid well were equipped with photobeams to detect operant responses. The events occurring in the behavioral apparatus were timestamped and synchronized online with the electrophysiological data using a Neuralynx Cheetah system (Bozeman, MT, USA). Odors were stored in glass vials and mixed 1:1 with clean air through odor-specific tubing to avoid cross-odorant mixing. Computer-controlled solenoid valves directed the airflow in and out of the odor port. Likewise, such valves controlled the tubing used to separately deliver quinine and sucrose solutions to the fluid well.

Training procedure
Animals were trained on a two-odor go/no-go discrimination task. Each session, two novel odors were presented to the rat in blocks of 5 + 5 pseudo-randomly ordered trials with positive (S+) and negative (S-) outcome-predicting stimuli. Positive and negative outcomes were sucrose and quinine solutions, respectively. When a nosepoke in the odor-port was detected and sustained for 500 ms, airflow through the port was switched from clean air to a mixture of clean air and odorant. Nosepokes were scored as correct if maintained for at least 750 ms after the initial 500 ms delay. After this combined period (1250 ms; ‘odor poke’), the trial light turned off, indicating to the animals that the nosepoke could be terminated. After the odor poke, rats could decide to make a ‘Go’ response towards the fluid well within 10 s after termination of the nosepoke. In the fluid well, another 1000 ms nosepoke response (‘fluid poke’) was required, forcing the animal to maintain its head above the
Chapter 4. NMDA-R IN OFC PLASTICITY

fluid well before the outcome was delivered. A Miss (failed go response) or Correct Rejection (no-go response to S-) was scored if the rat did not enter the fluid well within 10 s after terminating the odor poke. A Hit response was scored if sucrose was obtained, a False Alarm if quinine was obtained. Odor pokes and fluid pokes with durations shorter than the programmed minimum resulted in immediate termination of the trial, as indicated by offset of the trial light and start of the subsequent ITI. Responses during the ITI had no programmed consequences. A behavioral criterion was defined as scoring 85% correct responses (i.e. Hits and Correct Rejections) over a moving block consisting of the last 20 trials. When a rat reached the behavioral criterion, which occurred in the majority of sessions (N=15 out of 20) a reversal phase was introduced, in which the stimulus-outcome associations were switched (the previously positive odor was now coupled to quinine and the previously negative odor to sucrose solution). The number of trials to reach this behavioral criterion differed between rats, because some learned faster than others. To compare learning rates between pharmacological conditions across rats, we normalized the number of trials required to reach criterion per rat per session by the average number of trials required for all sessions performed by that rat (drug and control sessions).

Combidrive assembly
As compared to a previously designed tetrode-recording device (Gray et al., 1995; Gothard et al., 1996; Lansink et al., 2007), the novel “Combidrive” allows the individual vertical positioning of 13 electrodes as well as 1 microdialysis probe in the same region of interest. The design and validation of this instrument was previously described (van Duuren et al., 2007b), but the current study presents, to our knowledge, the first results targeting the manipulation of synaptic transmission and plasticity. The 14th tetrode was substituted by a CMA/7 custom-made microdialysis probe (CMA Microdialysis, Sweden), with a flexible shaft and a 6 kDa cutoff cuprophane membrane. The probe could be raised and lowered independently from the tetrodes. This also allowed replacement of the probe if its membrane appeared to have become impermeable (see below). The probe occupied a vertically oriented center canal inside an exit grid consisting of a ring of tetrodes (11 recording tetrodes and 2 references) at a distance of 500 microns, allowing the establishment of comparable drug diffusion gradients to the cells surrounding the tetrodes
The diameter of the exit grid was 2 mm.

(see Fig. 4.1B-D).
**Chapter 4. NMDA-R IN OFC PLASTICITY**

**Surgical procedure**

Prior to surgery, rats were anesthetized by intramuscular injection of 0.08 ml/100 g of Hypnorm (0.2 mg/ml fentanyl, 10 mg/ml fluanisone; VetaPharma, Leeds, UK), followed by 0.04 ml/100 g of Dormicur (5 mg/ml midazolam, s.c.; Roche, Woerden, the Netherlands) and mounted in a stereotaxic frame. Body temperature was maintained between 35 and 36°C using a rectal probe feeding back into a heating pad. After the cranium was exposed, six holes were drilled to accommodate six surgical screws, one of which served as electrical ground. A larger hole (approximately 2 mm radius) was drilled over left orbitofrontal cortex (OFC; 4.2 mm anterior, 2.8 mm lateral to bregma). The bundle holding the tetrodes and microdialysis probe was lowered onto the exposed cortex and the whole combidrive was subsequently fixed to the skull with surgical screws using dental cement. After anchoring the drive, the tetrodes and microdialysis probe were lowered 1 mm into the cortex. The animal received 0.1 ml/100 g of presurgical weight of a 10% Finadyne (flunixin meglumine 50 mg/ml; Schering-Plough Intervet, Boxmeer, the Netherlands) solution in saline subcutaneously as an analgesic. Furthermore, 2 ml/flank of saline was administered subcutaneously. Over the next 7 days, the animal was allowed to recover, with ad libitum food and water available. The recording tetrodes and microdialysis probe were lowered to their target region (upper border of OFC: AP = 4.2 mm, ML = -2.8 mm, DV = -3.0 mm; Paxinos and Watson, 2007). During the experiment, tetrodes were lowered further into the OFC in daily steps. The reference electrodes were lowered to -2.0 mm DV and adjusted to a position where no or very little spiking activity was recorded.

**Pharmacology**

On a daily basis, solutions used for reverse microdialysis were prepared freshly, with the exception of the solution containing 0.5 mM D-AP5 dissolved in aCSF (artificial cerebrospinal fluid; 149 mM NaCl; 4.6 mM KCl, 0.9 mM CaCl2, 0.5 mM MgCl2, 2 mM HEPES, 1.3 mM Glucose), which was prepared from a 10x stock solution. All solutions were buffered with HEPES to 7.4 pH and adjusted to approximately 300 mOsm. Solutions were perfused at a speed of 4.0 uL/min using a CMA/102 dual pump and 2.5 ml Hamilton stainless steel/glass syringes and needles through FEP tubing with a low internal volume (CMA microdialysis, Sweden). The commutator was retrofitted with a fluid
swivel (Quartz-lined dual channel swivel 375/D/22QM, Instech-Solomon, Plymouth, PA, USA). In about half of the recording sessions a solution of 0.5 mM D-AP5 in aCSF was perfused and in the other half a control solution of aCSF. The chemical flux passed the permeable part of the dialysis probe, extending 2 mm along the DV axis, at which point diffusion of the compounds into the brain was possible.

Validation of probe function

The microdialysis probes were chronically implanted, leaving the membranes vulnerable to glial encapsulation. In order to check probe functionality, we terminated each recording session with a 30-minute perfusion of a 2% lidocaine solution in aCSF (same composition as above, but with the addition of 69.25 mM lidocaine HCl (Sigma-Aldrich, Zwijndrecht, the Netherlands); NaCl concentration was reduced to 77 mM to maintain iso-osmolality to aCSF). This concentration of lidocaine is known to reversibly inhibit spiking of neurons recorded on nearby tetrodes (van Duuren et al., 2007b). The lidocaine perfusion was followed by a 30-minute washout period. Visual, online inspection of the aggregate spike rate of units that were being recorded served to estimate whether the lidocaine solution was able to reduce unit firing sufficiently. This procedure served to assess the condition of probe membrane permeability on that day. If lidocaine perfusion did not clearly reduce spiking, the recording session was discarded and a probe exchange procedure was initiated. This exchange procedure was shown to allow steady recordings before and after probe exchange (van Duuren et al., 2007b). In these experiments, we only resumed recording on the day following the probe exchange.

The lidocaine validation served a double purpose: next to being a probe permeability test, we used the lidocaine-induced reduction in firing rate as a metric to include or reject cells from further analysis. If lidocaine perfusion would fail to reduce the firing rate of a cell, it is unlikely that D-AP5 perfusion would strongly affect that cell either. Therefore, we only accepted those units into the subsequent analyses that showed a significant reduction and recovery of firing rate, during wash-in and wash-out of lidocaine, respectively. We compared spike rates in the last 10 one-minute bins before lidocaine, during lidocaine and during subsequent washout using two 2-sided t-tests, with significance set at p<0.05.
Chapter 4. NMDA-R IN OFC PLASTICITY

The flow speed in drug and control session was identical and we made sure that the infusion remained continuous during the recording sessions. Moreover, care was taken in the cluster cutting procedure that recorded single units remained stable throughout the session, as verified by inspecting spike waveform amplitudes plotted over time (cf. van Duuren et al., 2007). Online changes in perfusion medium (e.g. from aCSF to D-AP5 dissolved in aCSF) did not affect recording stability: the mean firing rate (FR) of units recorded before behavioral testing started did not significantly change across a switch in medium (Mean ± SEM log-transformed ratio FR\textsubscript{post}/FR\textsubscript{pre} = -0.028 ± 0.024; \(p = 0.23 / p = 0.97\), one-sample t-Test / sign test vs. 0).

Quantification of D-AP5 release into OFC tissue

To assess the extent to which D-AP5 is released into OFC, autoradiography was employed to quantify the spatial spread of the drug. Seven rats, weighing 437-504 g, received surgery as above. After the placement of the exit grid on the cortex, probes were lowered to the final site of infusion in 0.4 mm steps every three minutes (AP = 4.2 mm, ML = -2.8 mm, DV = -4.8 mm), under general anesthesia as above. After initial steady-state aCSF flow, rats were infused with 1.0 mM D-[\textsuperscript{3}H]AP-5 in aCSF (specific activity 60 Ci/mmol; Amersham Radiolabeling Service, GE Healthcare Life Sciences, Cardiff, UK) as above, for variable durations (2 rats for 30 min; 2 rats for 2 h; 3 rats as controls for 30 min). Control animals were continuously exposed to aCSF perfusion containing no radioactive material.

After perfusion, the microdialysis probe was raised and rats were sacrificed. Brains were removed from the skull and frozen in isopentane (Sigma-Aldrich, Zwijndrecht, the Netherlands), after which they were kept at -80°C. Brains were glued to the tray for sectioning using Tissue-Tek\textsuperscript{®} (Sakura Finetek Europe, Alphen aan de Rijn, Netherlands) and cut with a cryomicrotome (Leica Microsystems, Rijswijk, Netherlands) in 20 µm thick coronal sections at -20°C. The sections were placed into separate x-ray cassettes containing \textsuperscript{3}H-sensitive Amersham Hyperfilm (18x24 cm, GE Healthcare Life Sciences, Cardiff, UK), \textsuperscript{14}C glass slide standards (ARC, Saint Louis, U.S.A.) and \textsuperscript{3}H microscales (RPA510, GE Healthcare Life Sciences, Cardiff, UK). Exposure took place at room temperature for 12 days and was followed by routine film development.

For quantification, we analyzed the optical density of the developed
microfilms using the MCID 7.0 software package (InterFocus Imaging, Cambridge, UK). Briefly, we confirmed probe placement using coronal anatomical microphotographs. Next, we measured optical density at 500 µm ML on both sides of the probe in the plane of the probe track, and at plus and minus 500 µm displacement along the AP-axis, at ML coordinates matched to the probe track. These four points lie on a hypothetical circle in the horizontal plane that has the probe track as its center. Since the permeable part of the microdialysis probe spanned the entire DV axis of the OFC, diffusion into the OFC was assumed to occur from a hypothetical column instead of a point. Indeed, autoradiographical measurements confirmed a decay of the optical density in an elliptical (longer DV than ML axis, see Fig. 4.1B,C) rather than spherical fashion. This arrangement of probe and tetrodes allowed comparable diffusion gradients for tetrodes placed around the microdialysis probe at different depths.

Analysis of autoradiography data
D-[H]AP-5 is known to bind reversibly to rat brain membrane with a specificity of around 40%, depending on the brain area, with an IC$_{50}$ concentration of approximately 2.4 µM (Monaghan et al., 1984, 1988; Monaghan and Cotman, 1986). Early work by Davis et al. (Davis et al., 1992) indicated that the estimated free extracellular D-AP5 concentration, as measured by conventional microdialysis, is significantly less than the concentrations estimated through autoradiography methods: up to 1:30 of the estimated concentrations. This implies that most of the D-AP5 is either absorbed onto membranes or stored intracellularly. Thus, D-AP5 concentrations calculated from autoradiography measures should be divided by an estimated factor of 30 to conservatively approximate the free extracellular D-AP5 concentration available to NMDARs at synapses.

After routine development of our microfilms, D-[H]AP-5 optical density measurements taken 0.5 mm from the probe membrane after 30 minutes of perfusion corresponded to activity levels of at least 0.14 ± 0.01 µCi/µl (mean ± SEM) D-[H]AP-5; this level of radioactivity corresponds to 34% of probe D-[H]AP-5 radioactivity. In the rats that were perfused for 2 hours, this concentration had increased significantly to 0.26 ± 0.02 µCi/µl (one-sided t-test, p<0.05, Fig. 4.1B), corresponding to 59% of probe D-AP5 radioactivity. Applying the 1:30 ratio, at least 1-2% percent of the probe
Chapter 4. NMDA-R IN OFC PLASTICITY

D-AP5 concentration is thus estimated to be available in the extracellular space at 30 and 120 min after wash-in, respectively. In our recording experiments, this corresponds to concentrations in the range of 5-10 µM of D-AP5. This range of drug concentrations is known from slice studies to have major blocking effects at NMDARs, and to affect synaptic plasticity (Davies et al., 1981; Herron et al., 1986; Colino and Malenka, 1993; Cummings et al., 1996). To further illustrate the spread of radioactivity along the anterior-posterior axis, we made a composite image of coronal slices, aligned to the manually defined border between OFC and piriform cortex (Fig. 4.1C, purple line), reporting mean activity levels over a window with a ML width of 1.2mm. In conclusion, reverse microdialysis of D-AP5 is estimated to result in physiologically effective concentrations at recorded OFC neurons and to establish a limited spatial spreading throughout OFC and neighboring tissue.

Histology

After the final recording session, current (25 µA for 10 seconds) was passed through one lead per tetrode to mark the endpoint of the tetrode with a small lesion. The animals were deeply anesthetized with Nembutal (sodium pentobarbital, 60 mg/ml, 0.9 ml i.p.; Ceva Sante Animale, Maassluis, the Netherlands) and transcardially perfused with a 0.9% NaCl solution, followed by a 4% paraformaldehyde solution (pH 7.4 phosphate buffered). Following immersion fixation, coronal sections of 40 µm were cut using a vibratome and stained with Cresyl Violet to reconstruct tetrode tracks and localize the endpoints. Histological verification of the tetrode endpoints and recording tracks showed that all recordings were performed from +3.6 to +4.6mm AP of Bregma, from -2.0 to -3.6mm ML and from -3.5 to -4.8 DV.

Electrophysiology and spike sorting

Using tetrodes (Gray et al., 1995), we recorded neural activity with a 64-channel Cheetah setup (Neuralynx, Bozeman MT). Signals were passed through a unity-gain pre-amplifier headstage, a 72-channel commutator (Dragonfly, Ridgeley, West Virginia, USA), amplified 5000x and bandpass filtered between 600-6000 Hz for spike recordings. One ms epochs of activity from all four leads were digitized at 32kHz if a signal on any of the leads of a tetrode crossed a pre-set threshold. Local field potentials recorded on all tetrodes were amplified 1000x, continuously sampled at 1874 Hz, and
bandpass filtered between 1-475 Hz. Spike trains were sorted to isolate single units using a semi-automated clustering algorithm followed by manual refinement (KlustaKwik, Ken Harris and MClust 3.5, Dave Redish). Automated and manual clustering of spikes was done using the waveform peak amplitude, area, squared amplitude integral and the first three principal components. Clusters were accepted as single units when having no more than 0.1% of inter-spike intervals shorter than 2 ms.

Analysis of behavioral correlates
We constructed peri-event time histograms (PETHs) synchronized on task events (i.e., on- and offset of odor sampling, fluid well entry, reward delivery) to evaluate behavioral correlates of firing-rate changes. Firing rates in time bins (100 ms) around the event of interest were each compared against firing rates in 5 control bins from the ITI on a trial-by-trial basis to exclude within-session drift of firing rate as confounding factor and tested for significance using the non-parametric Wilcoxon’s matched-pairs signed-rank (WMPSR) test (p<0.01). Binned firing rates were considered significantly modulated in relation to the task event only if the test bin of interest differed significantly from all 5 baseline bins (van Duuren et al., 2007a). Cells were classified into four categories based on the task period in which they exhibited significant firing-rate changes (for odor sampling: change in relation to odor onset and during odor delivery; movement: change after odor offset but before fluid poke onset; waiting: change after fluid poke onset but before outcome; outcome: change following application of sucrose or quinine solution, see Table 2).

ROC analysis
To quantify the ability of firing rates to discriminate between the S+ and S- conditions, we performed an ROC analysis (cf. Green and Swets, 1966; Histed et al., 2009) on the firing rates that were obtained by convolving spike trains with a 250 ms Hann window. Let A be the area under the ROC curve, ranging from 0 to 1. The quantity

\[ D_{\text{raw}} = |A-0.5| \]  

(Eq 1)

is a measure of discrimination power, strictly ranging from 0 to 1/2. The measure \( D_{\text{raw}} \) is a positively biased statistic (see e.g. Histed et al., 2009), due
to the convexity of the absolute value operator in the expression |A-0.5|. To remove this positive sampling bias, we computed $D_{raw}$ also for permuted data in which the trials were randomly assigned to either the S+ or S- condition, while leaving the number of trials per condition as in the original data (as in Histed et al., 2009). The shuffled ROC area, $D_{shuff}$, was then determined by averaging $D_{raw}$ across 5000 permutations. The measure of discrimination power used for Figs. 4.3 and 4.9 was then defined as the quantity

$$D_{corrected} = D_{raw} - D_{shuff}$$

(Eq 2)

The equality $D_{corrected} = 0$ indicates the absence of discrimination power, while $D_{corrected} = 0.5$ indicates maximum discrimination power. Note that negative values can incidentally occur due to under-sampling, e.g. if trials have very similar firing rates between conditions, although the expected value of $D_{corrected}$ strictly lies between 0 and ½ such that $[D_{corrected} + 1/2]$ is equal to the measure used by Histed et al., (2009).

For the time-resolved $D_{corrected}$ (Fig. 4.3A), we reported bootstrapped means of $D_{corrected}$ values per pharmacological condition, obtained using spike train segments selected with 200 ms sliding windows. The histograms of $D_{corrected}$ values (Fig. 4.3B, 4.9) show the underlying $D_{corrected}$ distributions at time windows during the acquisition and reversal phase for the intertrial interval (-1 to -0.5 s before odor onset), and during task periods (odor sampling [0.5 to 1 s after odor onset], early and late movement period [0 to 0.5 s after odor offset and -0.5 to 0 s before fluid well entry, respectively] and waiting period [-1 to 0 s before outcome delivery]).

**Consistency of firing rate selectivity**

To test whether units retained the direction of their S+/S- firing rate selectivity from acquisition to reversal, we performed the following analyses: We first selected all cells that expressed some degree of selectivity for the S+/S- condition in the acquisition phase (criterion: $D_{corrected, acq} > 0$). We then assessed whether the S+/S- selectivity was consistent between acquisition and reversal phase, by computing a quantity that we call the *consistency of selectivity*, defined as

$$C = \text{sign}(A_{acq} - 1/2) \times \text{sign}(A_{rev} - 1/2)$$

(Eq 3)

110
Here, $A_{\text{acq}}$ is the raw ROC value computed for the acquisition phase, where $A_{\text{acq}}>0.5$ and $A_{\text{acq}}<0.5$ reflect higher and lower firing rates for the first and second odor, respectively. The quantity $A_{\text{rev}}$ is defined in the same way but now for the reversal phase. If the consistency of selectivity $C$ equals 1, then a unit preserves its (odor) selectivity from acquisition to reversal, whereas $C$ equal to -1 indicates that firing rate selectivity flips from acquisition to reversal, following the change in actual outcomes.

The consistency of selectivity measure $C$ has the strong disadvantage that it does not incorporate to what degree individual cells are selective in the reversal phase ($D_{\text{corrected, rev}}$, Eq. 2). For units that have small $D_{\text{corrected, rev}}$ values, measured values of $C$ are particularly unreliable. To take this degree of firing rate selectivity into account, we computed a measure called the $D_{\text{corrected}}$ consistency, defined as $[C \times D_{\text{corrected, rev}}]$, where $C$ is defined in Eq. 3. We computed $D_{\text{corrected}}$ consistency for each unit separately. An average value of $D_{\text{corrected}}$ consistency that is positive indicates that there is preservation of firing selectivity from the acquisition to reversal phase. If the average value of the $D_{\text{corrected}}$ consistency is negative, this indicates that firing rate selectivities flip from acquisition to reversal. An average value of $D_{\text{corrected}}$ consistency around zero indicates a mixture of maintenance and flips of firing selectivity. The $D_{\text{corrected}}$ consistency values can also be compared to the $D_{\text{corrected, rev}}$ values. An average value of $D_{\text{corrected}}$ consistency smaller than $D_{\text{corrected, rev}}$ indicates that at least some units flip their firing rate selectivities.

The $D_{\text{corrected}}$ consistency measure has the disadvantage that the $D_{\text{corrected, rev}}$ value can occasionally turn negative in case of low trial numbers (as $[D_{\text{corrected, rev}} = D_{\text{raw, rev}} - D_{\text{shuffled, rev}}]$, Eq. 2). This can sometimes result in the $D_{\text{corrected}}$ consistency being larger than the $D_{\text{corrected, rev}}$ value, as in Fig. S4.10C. To solve this problem, we also computed a quantity called the $D_{\text{raw}}$ consistency, defined as $[C \times D_{\text{raw, rev}}]$, where $D_{\text{raw, rev}}$ was defined in Eq. 1. The advantage of this measure is that the inequality $[D_{\text{raw}} \text{ consistency} < D_{\text{raw, rev}}]$ strictly holds, as $D_{\text{raw, rev}} > 0$. Because the $D_{\text{raw}}$ value is biased by the number of trials (being larger for few trials), one cannot make fair statistical comparisons between drug conditions and acquisition vs. reversal phase using $D_{\text{raw}}$ consistency and $D_{\text{raw}}$. However, one can readily compare these $D_{\text{raw}}$ consistency values if they are based on the same number of trials (i.e., between $D_{\text{raw, rev}}$ and $D_{\text{raw}}$ consistency, Fig. 4.11).
Chapter 4. NMDA-R IN OFC PLASTICITY

Pseudo discrimination scores
Because the ROC is per definition computed over a set of trials, it cannot be computed for an individual trial. However, it is possible to determine the contribution of a single trial to the ROC that is computed over a set of trials. To obtain a measure of this contribution, we computed single-trial pseudo-discrimination scores, an approach similar to e.g. pseudo-correlations or pseudo-coherence values (Womelsdorf et al., 2006, 2007, Fig. 4.4). Again, let $A$ be the standard ROC area based on $N$ trials ($0 \leq A \leq 1$). For trial $i$, let $A_{not-i}$ be the ROC area based on $N-1$ trials (i.e., excluding the $i$th trial). For the $i$th trial, we then compute the pseudo ROC area $A' = N A - (N-1) A_{not-i}$. The inequality $A' > A$ indicates that the $i$th trial contributes positively or negatively to the rectified ROC area $D_{raw} = |A-1/2|$ if $A>0.5$ or $A<0.5$, respectively. We therefore define the pseudo discrimination score as

$$D_{pseudo} = (A' - A) \cdot \text{sign}(A-1/2)$$  \hfill (Eq 4)

where \text{sign} is the sign function. If the pseudo discrimination score is positive, the $i$th trial contributes positively to the $D_{raw}$ and to $D_{corrected}$. If the pseudo discrimination score is negative, then the $i$th trial contributes negatively to the $D_{raw}$ and $D_{corrected}$. In other words, $D_{pseudo}^i$ represents the contribution of trial $i$ to the overall discrimination measure. A positive or negative discrimination score imply that including or removing the $i$th trial respectively enhances or reduces the overall discrimination. The pseudo discrimination score hence reflects to what extent the overall firing-rate preference of a cell for S+ over S- trials is expressed in an individual trial. If there is no change in $D_{corrected}$ across trials, then the expected value of $D_{pseudo}^i$ equals zero for a given trial. This analysis allows the assessment of the contributions of the S+ and S- spike trains over trials (e.g. in S+ analysis only one S+ trial was removed, while keeping all other S+ and S- trials).

To model the dependence of the pseudo discrimination score on trial number, non-linear regression was performed on the pseudo discrimination score data for both the S+ and S- trials, using a model with a constant, linear and exponential term: $y = a + bx + e^{cx}$, where $x$ is the trial number and $y$ is the pseudo discrimination score. Using an iterative procedure, we fitted these three parameters to the pseudo discrimination score group data (Fig. 4.4C-D) and computed 95% confidence intervals on these parameters to
determine significance at P<0.05.

**Statistical analysis of ROC and pseudo discrimination scores using bootstraps**

A potential source of systematic variance in our results is inter-subject variability, because more cells could have been recorded from a given rat for one than the other pharmacological condition. To remove this source of variance, we performed the following procedure, to which we henceforth refer as ‘stratified bootstrap’ procedure: We used N=5000 bootstrapped approximations of the statistic of interest, e.g. $D_{\text{corrected}}$ or the pseudo discrimination score. Importantly, in each bootstrap repetition, we randomly drew equal numbers (N=50, with replacement) of neurons per rat (from the pool of all cells that were recorded for a given rat in a pharmacological condition) for the drug and control condition. We call such a bootstrap that stratifies the number of cells across pharmacological conditions per rat a ‘stratified bootstrap’. Given the two drug conditions with means $X_{\text{ACSF}}$ and $X_{\text{AP5}}$ for the statistic under consideration, let $\mu$ be the absolute difference in bootstrap means: $\mu = |X_{\text{ACSF}} - X_{\text{AP5}}|$ and $\sigma$ the pooled standard deviation of the bootstrap distributions. Statistical significance was then determined by comparing $Z = \mu/\sigma$ to the normal distribution. We refer to this test as “Bootstrap test”.

**Spectral analysis of LFP**

To compute the LFP power spectrum, we first zero-padded the LFP signals that were obtained from the odor sampling period to 5 sec., in order to ensure a constant frequency resolution (of 0.2 Hz) across trials. We then Fast-Fourier-Transformed the Hann-tapered LFP signals, obtaining the raw LFP power spectrum. Next the relative LFP power spectrum was computed by dividing the raw LFP power spectrum by the total power in the interval [2, 200] Hz. The relative power spectrum was then log-transformed.

Dependence of LFP power on trial number

LFP power was first normalized by dividing by the average LFP power over the first 50 trials, for every channel separately. We then computed the average normalized LFP power for every trial number. Linear regression analysis of average normalized LFP power onto trial number was performed. Differences in regression slopes were assessed with a Multiple Comparison
Chapter 4. NMDA-R IN OFC PLASTICITY

Corrected [MCC] Permutation Test on T-statistics (Bullmore et al., 1999; Maris et al., 2007)

Spike-LFP phase-locking

To compute spike-LFP phase-locking, we computed the pairwise phase consistency (PPC; Vinck et al., 2010, 2011). For a given spike and frequency $f$, we computed the instantaneous spike-LFP phase by Hann-tapering an LFP segment centered around the spike of length $5/f$ sec. Only spike-LFP pairs were included for which the isolated unit was recorded on a different electrode than the LFP. We then computed the PPC across spike-LFP phases. The PPC is an estimator of the squared resultant length (phase-locking value) that is not biased by the number of spikes, but also not by non-Poissonian history effects within spike-trains (Vinck et al., 2010b, 2011a), and its expected value ranges from 0 (no phase-locking) to 1 (maximum phase-locking). The PPC was defined as follows (called PPC 1 in Vinck et al., 2011). For a given frequency, let $\theta_{j,m}$ be the spike-LFP phase for the $j$-th spike in the $m$-th trial ($m = 1, ..., M$), which contains $N_m$ spikes; $l$ denotes a trial different from $m$. The PPC was then defined according to eq. 3.1 in Vinck et al. (2011) as

$$
\text{PPC} = \frac{\sum_{m=1}^{M} \sum_{l>m} \sum_{j=1}^{N_m} \sum_{k=1}^{N_l} \left( \sin(\theta_{j,m}) \sin(\theta_{k,l}) + \cos(\theta_{j,m}) \cos(\theta_{k,l}) \right)}{\sum_{m=1}^{M} \sum_{l>m} N_m N_l} \cdot \frac{1}{M}
$$

The PPC quantifies the average coincidences of pairs of spikes from separate trials in the spike-LFP phase domain (Vinck et al., 2010b, 2011a). The PPC was computed for every neuron separately (only neurons were included containing at least 50 spikes, as in van Wingerden et al. (van Wingerden et al., 2010a, 2010b). To compile group averages, we used the same stratification bootstrap method ($N = 5000$) as used for the ROC analysis, drawing an equal number of cells per rat for both the drug and control condition, with replacement. Similar to the ROC analysis, we then estimated the mean and standard deviation of the bootstrap distributions and compared the resulting T-statistic to the normal distribution.
RESULTS

We recorded 623 isolated single units from four rats in 20 counterbalanced sessions (number of sessions for drug/control = 10/10) using a modified microdrive that held 12 tetrodes arranged concentrically around a microdialysis probe (Fig. 4.1D). Histological verification indicated that most recordings were from ventral and lateral orbitofrontal (VO/LO) and agranular insular (AI) cortex with some spread into dorsolateral orbitofrontal (DLO) cortex (Fig. 4.1A). For each rat, recordings were obtained under both drug and control conditions. However, within a single session, only one condition was applied. In drug sessions, we used continuous reverse microdialysis to apply a 0.5 mM solution of D-2-amino-5-phosphonopentanoate (D-AP5), a competitive NMDAR blocker, dissolved in aCSF. A separate autoradiography
experiment with $^3$H-D-AP5 confirmed that the concentration of D-AP5 in OFC was sufficient to antagonize NMDARs (Fig. 4.1 B-C). Infusions were done unilaterally to minimize the chance of inducing behavioral effects, which could confound the interpretation of electrophysiological results if present.

**NMDA receptor blockade and behavioral performance**

Rats performed a two-odor, go/no-go discrimination task, with novel odor-outcome associations for each session (Fig. 4.2A-B; Schoenbaum et al., 1998; van Duuren et al., 2007; van Wingerden et al., 2010a, 2010b). Task acquisition was manifested by the emergence of ‘No-go’ and ‘Go’ responses to the odors predicting a negative (S- condition; ‘correct rejections’) and positive outcome (S+ condition; ‘hits’), respectively. The acquisition phase of the task was terminated when rats reached a behavioral criterion (85% correct trials, i.e. ‘hits’ + ‘correct rejections’, in a moving 20-trial block), after which a reversal phase followed, in which the previously presented stimulus-outcome pairings were switched. We first examined overall task performance, defined as the average number of trials to reach criterion, normalized per rat to the average number of trials to criterion for all drug and control sessions for that rat. Overall performance did not differ between control and drug sessions (mean ± SEM; control: 103%±8.6%, drug: 96.7%±9.6%, two-sided t-test n.s., Fig. 4.2C). Reaction time (RT), i.e. the time between odor poke termination and subsequent fluid well entry, decreased with acquisition trial number in the S+ condition for both the control (Spearman’s rho = -0.24, p<0.01) and drug condition (rho = -0.17, p<0.05; difference in slopes between conditions n.s., Fisher’s Z-test: p = 0.31), but not in the S- condition (‘false alarm trials’; p-values: 0.82 [aCSF] and 0.24 [D-AP5]). RT was faster for hits than false alarm trials, both for control and drug sessions (p<0.001 and p<0.05, respectively; Mann-Whitney U-test). No significant difference in RT between control and drug sessions was detected, neither for hits nor false alarms (p = 0.07 and p = 0.23, respectively; Mann-Whitney U-test). Altogether, the absence of significant behavioral differences between the drug and control condition for the task acquisition phase, indicates that electrophysiological comparisons between these two conditions can be made in a comparable behavioral context.

This finding contrasts with the early reversal phase, where we did observe an effect of unilateral D-AP5 infusion. Here, the mean Z-scored RT
after reversal differed significantly from the last 10 trials before reversal for both S+ to S- and S- to S+ transitions in control (p<0.01, Mann-Whitney U test, Fig. S1), but not drug sessions (p = 0.28, p = 0.76 respectively). Direct comparisons between RTs indicated that RT for aCSF and D-AP5 sessions
Chapter 4. NMDA-R IN OFC PLASTICITY

did not differ for the last 10 trials before reversal (p>0.05 for both S+ and S- trials, Mann-Whitney U-test). Post-reversal, however, we found significant differences in Z-scored RT between pharmacological conditions for both S+ (ACQ) trials, now S- and S- (ACQ) trials, now S+ (p<0.001 and p<0.05, respectively, MWU-test).

Effect of NMDAR blockade on firing rates

Out of the 623 recorded cells, 281 (117 for D-AP5, 164 for aCSF) units were included for further analysis because of their responsiveness to perfusion (see Experimental Procedures). Unless stated otherwise, all further analyses pertain to the acquisition phase of the task. After exclusion of putative fast-spiking interneurons (N_{aCSF} = 20; N_{D-AP5} = 7) based on waveform characteristics (van Wingerden et al., 2010b), we did not detect a significant difference in the mean raw firing rate of putative pyramidal cells between the control and drug condition for the ITI (inter-trial interval) baseline period (FR_{aCSF} mean ± SEM: 2.35 ± 0.33 Hz, FR_{D-AP5}: 1.78 ± 0.32 Hz, n.s., Mann-Whitney U-test; Fig. 4.2D), and the three task periods leading up to the outcome (odor sampling, locomotion from odor port to fluid well, waiting period; Table 1). However, for all of these three task periods we found increased firing rates relative to baseline for the drug (across periods: mean ± SEM = 138 ± 9.5%, p<0.01, Mann-Whitney U-test; Fig 4.2E), but not for the control condition (102 ± 3.7%).

Effect of NMDAR blockade on discriminative power of firing rates

To examine whether D-AP5 affected the firing rate discrimination between the S+ and S- condition, we performed an ROC (Receiver Operator Characteristic) analysis (cf. Green and Swets, 1966; Histed et al., 2009). For each unit, we examined to what extent firing rates in fragments of 200 ms discriminated between S+ and S- by computing the shuffle-corrected ROC area, called $D_{corrected}$ (Fig. 4.3): an index ranging from 0 (no discriminative power) to 1/2 (maximum discriminative power; negative numbers incidentally occur because of limited sampling). We then averaged $D_{corrected}$ across units, while balancing, for a given rat, the number of cells entered in the analysis across pharmacological conditions. In the acquisition phase, D-AP5 caused a decrease in $D_{corrected}$ values for the odor period (Fig. 4.3; P<0.01, Bootstrap test with Bonferroni correction). In this phase, no significant drug effects were
In the reversal phase D-AP5 significantly reduced $D_{\text{corrected}}$ values during the odor as well as early and late movement phases. Units’ $D_{\text{corrected}}$ scores in the reversal phase may reflect the sign (direction) of their acquisition-phase firing rate selectivity, or a reversed selectivity. In fact, maintenance of cue selectivity across cue-outcome reversal has been linked to faster reversal learning (Stalnaker et al., 2006; Schoenbaum et al., 2007). To assess the consistency of firing rate selectivity, we applied a sign function to the $D_{\text{corrected}}$ calculation. We found that, after reversal, firing rate selectivity was preserved especially in the odor sampling phase of control, but not drug sessions.

Figure 4.4 Evolution of discrimination scores over acquisition trials

(A-B) Bootstrapped mean of pseudo-discrimination scores for the acquisition odor phase against S+ (A) and S- (B) trial number. Positive pseudo-discrimination scores indicate that a trial contributed positively to overall ROC area. Shading indicates SD of bootstrap distribution. Horizontal purple lines indicate significant differences between group means (p<0.05, Bonferroni corrected, Bootstrap test). (C) Best non-linear fit (dashed lines) to the S+ group data (solid lines, as in panel A). (D) as in (C), but now showing fits to the S- group data of (B). (E) Bootstrapped mean difference in pseudo-discrimination scores between the first and the last trial of the acquisition phase (± SD across bootstraps). *, **: p<0.05, p<0.01; Bootstrap test. (F) as in (E), but now for the S- odor period.
Chapter 4. NMDA-R IN OFC PLASTICITY

whereas firing rate selectivities showed a mixture of maintenance and flips for the movement and waiting phases (Fig. 4.10, 4.11).

The observed effects of D-AP5 are unlikely to be a consequence of changes in the prevalence of firing-rate correlates. A unit was defined as having a firing-rate correlate for a given task period if its firing rate in that period differed significantly from the ITI firing rate. The distribution of firing rate correlates did not significantly differ between control and drug conditions across task periods (Table 4.2; chi-square test; df = 4; p = 0.64).

**Plasticity in discriminative power of firing rates**

One route by which D-AP5 may impact discriminatory firing is through the impairment of NMDAR-dependent, long-term synaptic plasticity, which may be required for neurons to develop stimulus-outcome discrimination across learning trials. Alternatively, NMDARs may acutely support discriminatory firing because of their slow-EPSP contributions. If the effects of D-AP5 are mediated via long-term plasticity, they should gradually become more pronounced across trials. To investigate how effects of D-AP5 on outcome-selective firing patterns develop across trials, we examined single-trial contributions to ROC discrimination scores using a leave-one-out procedure, yielding pseudo discrimination (PD) scores per trial (see Experimental Procedures). A positive or negative PD score for a given trial indicated that inclusion of the trial had a positive or negative effect, respectively, on the overall ROC. PD scores were averaged across cells, separately for the odor, movement and waiting period. For both the S+ and S- trials in the odor period, we found an upward trend in average PD score over trials for the control condition, with higher PD values compared to the drug condition on later trials (p<0.05, Bootstrap test against shuffled data, Fig. 4.4A-B). To quantify the magnitude of changes in PD scores across trials, we first computed the mean difference in average PD scores between the first and last trial. For both S+ and S- trials, this difference was higher than zero for the control, but not drug condition (Bootstrap test; p<0.05, Fig. 4.4E-F). The mean difference score was higher for control than drug units, for both trial types (p<0.01, Bootstrap test).

Second, to model the relationship between mean PD score and trial number, we performed a regression analysis with a linear and exponential term. Best fits were obtained by iterative fitting (Fig. 4.4C-D). For both the
S+ and S- condition, linear and exponential parameters were significantly different from zero for the control (i.e., the 95% confidence interval for the fitted parameters did not contain zero), but not for the drug condition. Finally, we note that during the movement and waiting periods of control and drug sessions, the population averages of PD scores did not show a clear upward trend across trials, indicating an absence of significant plasticity of discrimination between trial types in these periods. Thus, with learning, the discriminability of spike train responses to odor stimuli slowly increased, and selectively so for the odor period. This process depends, at least in part, on NMDAR function. Overall, we found no significant effect of D-AP5 on early learning trials, as would otherwise have supported a function of NMDARs on acute processing by slow EPSP contributions.
Chapter 4. NMDA-R IN OFC PLASTICITY

Effect of NMDAR blockade on rhythmic synchronization

In addition to affecting firing rates and discriminative coding, NMDARs may well regulate rhythmic mass activity as visible in LFPs, and concomitant entrainment of OFC neurons to these signals. We focused on odor sampling because of the strong changes in ROC discrimination scores during this period, and our previous finding of strong gamma- and theta-band synchronization during stimulus processing (van Wingerden et al., 2010b). In LFP signals, we found that D-AP5 induced a broad-band increase in relative power for the theta-band as well as frequencies above ~20 Hz and a concurrent decrease in low-frequency power (P<0.05, Fig. 4.5, Multiple Comparison Corrected [MCC] Permutation Test on T-statistics; Bullmore et al., 1999; Maris et al., 2007). We confirmed our previous finding that LFP gamma-band power increases with trial number and is predictive of learning (van Wingerden et al., 2010b). A similar increase in LFP gamma power with trial number was observed for the drug condition (Fig. 4.12). However, we found theta power to be negatively correlated with trial number in the drug, but not control condition (significant difference in slopes between D-AP5 en aCSF; P<0.05; MCC Permutation test, Fig. 4.12).

To investigate whether D-AP5 affects local phase-synchronization of single units, we computed the spike-LFP pairwise phase consistency (PPC; Vinck et al., 2010b, 2011). D-AP5 had a three-fold effect (Fig. 4.5C-D, p<0.05, MCC Permutation Test on T-statistics). First, it strongly increased theta locking (~10Hz) by about 100%. Secondly, a beta (20-25 Hz) rhythm emerged, which was absent in the control condition. Thirdly, it increased spike-LFP phase-locking in the supra-gamma range (110-160 Hz).

Finally, we tested whether D-AP5 altered the relationship between neuronal discrimination scores and spike-LFP phase-locking patterns. For the 0.5-1.0 sec. period of odor sampling (during which ROC values peaked) we correlated the unit’s time-resolved $D_{\text{corrected}}$ ROC values with their spike-LFP PPC values, separately for D-AP5 and aCSF. Differences in Spearman-rank correlations between the drug and control condition were observed in the theta and supra-gamma range (Fig. 4.6A, P<0.05; MCC Permutation test). For the control condition, we found that spike-LFP theta PPC positively predicted $D_{\text{corrected}}$ with significant correlations peaking (Fig. 4.6B; P<0.05, MCC Permutation test on difference in Spearman rho’s,) around the time when the $D_{\text{corrected}}$ values peaked (0.5-1s after odor onset, Fig. 4.3). However,
in the same time window D-AP5 induced a negative correlation between \(D_{\text{corrected}}\) and supra-gamma PPC values (Fig. 4.6A,C).

**DISCUSSION**

In conditions where a unilateral NMDAR blockade in rat OFC did not affect task acquisition behavior and modestly increased task-related firing rates relative to baseline, we showed that this receptor plays a significant role in neural representations discriminating between stimulus-outcome conditions and plastic changes in firing patterns associated with learning these representations. Especially during odor processing and decision-making the capacity of OFC neurons to discriminate between cues predictive of different outcomes was impaired by NMDAR blockade. In addition, NMDAR blockade increased local rhythmic synchronization, as indexed by spike-LFP phase-locking, particularly in the theta (~10 Hz), beta (20-30 Hz) and high-frequency range (110-150 Hz). Finally, we found a positive relationship between theta phase-locking and neuronal discrimination scores under control conditions, which was abolished by NMDAR blockade.
Chapter 4. NMDA-R IN OFC PLASTICITY

One concern when examining drug effects on neurophysiological correlates of cognitive processes is that the drug may affect behavior, which could in turn affect firing patterns in OFC known to represent relevant behavioral task components (Schoenbaum et al., 2009; Pennartz et al., 2011a). Bilateral infusion of NMDAR antagonist in OFC has been shown to increase impulsive responding and impair reversal learning (Bohn et al., 2003b). Therefore we chose unilateral drug application, and indeed found that performance scores and reaction time (RT) during task acquisition did not significantly differ between pharmacological conditions (in line with e.g. De Bruin et al., 2000). However, upon transition to the reversal phase, RT for Hit and False Alarm trials showed a characteristic flip in behavioral responding according to the reversed task rule in control sessions, while such a flip was absent in drug sessions (Fig. 4.8). Changes in RT in this type of task are thought to depend on OFC function (Bohn et al., 2003a; Schoenbaum et al., 2003a), particularly during the reversal phase in which NMDARs have been implicated by a previous study (Bohn et al., 2003b). Here, we show that unilateral blockade of NMDARs produces a comparable deficit in shaping discriminatory behavior according to updated task rules.

Firing-rate effects of NMDA receptor blockade in comparison to previous studies

Previous work indicated that systemic injections of a nonspecific, open-channel NMDAR blocker (MK-801) in freely moving rats leads to increments and decrements in average basal firing rates in putative pyramidal cells and fast spiking interneurons, respectively (Jackson et al., 2004; Homayoun and Moghaddam, 2007, 2008). Here we perfused a competitive NMDAR antagonist (D-AP5) directly into the OFC, and found that putative pyramidal cell firing rates during the ITI did not significantly differ between drug and control sessions (Fig. 4.2D). However, drug infusion induced a significant increase in relative firing rate during the various task stages (Fig. 4.2E). Thus, the results from the current and previous studies differ insofar as we did not observe an overall firing-rate elevation during the ITI (as was the case in Jackson et al., 2004; Homayoun and Moghaddam, 2007, 2008). This difference may stem, first, from differential effects of systemic versus local OFC applications. Systemic injections may affect various stages of processing afferent to the OFC, e.g. in the mediodorsal thalamus, piriform
cortex and basolateral amygdala. Secondly, in contrast to D-AP5, MK-801 acts as a dissociative anesthetic and impairs normal neural functioning, sometimes even causing cell damage and neuronal swelling (Olney et al., 1989). Whereas previous studies reported behavioral stereotypy induced by MK-801, we showed that behavioral patterns were not affected by local D-AP5 in the acquisition phase.

When considering a simplified circuit diagram of OFC pyramidal cells and interneurons (Fig. 4.7), we may tentatively explain the trend towards lower baseline firing rates of putative pyramidal cells under D-AP5 by a reduction in recurrent OFC network activity due to low afferent stimulation in the absence of task-oriented behavior. When the animals were engaged...
Chapter 4. NMDA-R IN OFC PLASTICITY

In the task, no significant differences in absolute firing rates between pharmacological conditions were detected. These findings do not support a general pyramidal neuron ‘hyperexcitability’ via a local reduction of NMDAR-mediated GABAergic inhibition (Homayoun and Moghaddam, 2007, 2008; Lisman et al., 2008), in line with a recent slice study indicating only a minor role for NMDARs in fast-spiking interneuron activity (Rotaru et al., 2011).

These findings are relevant for evaluating current hypotheses on schizophrenia. An involvement of NMDARs in schizophrenia is suggested by pharmacological studies of human volunteers subjected to non-specific NMDAR antagonists, such as ketamine, and recent post-mortem studies on schizophrenic patients (Malhotra et al., 1997; Lahti et al., 2001; Krystal et al., 2003; Gilmour et al., 2012). The NMDAR hypofunction theory proposes that schizophrenia is associated with a reduction of NMDAR-mediated currents at pyramidal-interneuron synapses, resulting in low activity of interneurons and disinhibition of pyramidal neurons (Olney et al., 1999; Lewis and Moghaddam, 2006; Homayoun and Moghaddam, 2007; Lisman et al., 2008). Our data indicate that NMDAR blockade-induced hyperactivity in OFC does not arise strictly from local mechanisms, because a blockade did not significantly affect absolute firing rates of putative pyramidal neurons. Such hyperactivity likely arises from global interactions between OFC and other areas. Our data further suggest that the reduction in neuronal cue-outcome selectivity and plasticity could contribute to impairments in OFC-dependent sensory gating and cognitive function as reported in schizophrenic patients (Krystal et al., 2003; Lisman et al., 2008). Finally, consistent with theories regarding schizophrenia as a disorder of inter-areal connectivity (Stephan et al., 2009; Lynall et al., 2010), our data show that local NMDA hypofunction causes marked changes in spike-field phase-synchronization, which may result in global dysconnectivity between brain areas (Uhlhaas et al., 2008).

Orbitofrontal NMDA receptors in discriminating stimulus-outcome patterns

In line with Schoenbaum et al. (1998, 1999), who demonstrated firing-rate selectivity in OFC for stimuli predictive of positive versus negative outcome, we found that during acquisition the electrophysiological S+/S- discrimination scores were significant during the entire task sequence from odor sampling to outcome delivery, both under drug and control conditions (Fig. 4.3). D-AP5 diminished the discriminatory power of single units only during odor
sampling. Under aCSF perfusion, the discrimination score during odor sampling increased over trials, due to adaptive changes in spike patterns across both S+ and S- trials (Fig. 4.4). NMDAR blockade hampered the trial-dependent plasticity of discrimination scores across learning during the odor phase. The reduction in discrimination scores by NMDAR blockade cannot be attributed to a difference in absolute firing rates, because these did not differ significantly between pharmacological conditions for any behavioral period (Table 4.1). Upon reversal, under D-AP5 perfusion, units lost their pre-reversal selectivity during cue sampling, while this selectivity was maintained for control units (Fig. 4.10-11). Maintenance of neuronal cue selectivity has been linked to faster reversal learning (Stalnaker et al., 2006; Schoenbaum et al., 2007). Here we show that this maintenance of neuronal cue-selectivity is NMDAR-dependent. Altogether, these results support the conclusion that NMDAR blockade renders firing patterns of OFC units less plastic and thus less robust in their discriminatory capacity during task acquisition and

Figure 4.8 Changes in Reaction Time from the pre- to post reversal phase

(A) Reaction time (i.e., times from odor poke termination to fluid well entry; RT) were Z-scored relative to the RT average of all pre-reversal trials. Bar histograms are shown for S+ trials, where S+ denotes the odour coupled to reward in the acquisition phase, hence S+ acq (pre-reversal: Hits; post-reversal: False Alarms) in control (blue) and drug (red) sessions, pre- and post-reversal. ACQ: mean Z-scored RT over last 10 trials before reversal. REV: mean Z-scored RT in first 10 trials of the reversal period. (B) As in A-B, but now showing S- trials (pre-reversal: False Alarms, post-reversal: Hits). For the control condition, the changes are in the direction predicted by the change in rules linking stimuli and outcome, with RTs to the odor now predicting sucrose decreasing and RTs to the odor now predicting quinine increasing. **, ***: p<0.05, p<0.01, p<0.001, Mann-Whitney U test
Chapter 4. NMDA-R IN OFC PLASTICITY

reversal, which may compromise the efficiency of OFC signaling during learning.

During acquisition, the discriminatory power of OFC neurons was strongly affected only in the odor period (Fig. 4.3A). At first sight, these findings contrast with the reproducibly reported coding of outcome expectancy parameters by OFC neurons during post-decisional anticipation and processing of outcomes (Schoenbaum et al., 1998, 1999; van Duuren et al., 2008; O’Neill and Schultz, 2010). Because the present study combined recording with local intervention, it presents a strong case for an NMDAR-dependent mechanism in OFC for pre-decisional processing of stimulus information, coupled to the retrieval of odor-associated values predicting future outcome. Do NMDARs primarily support learning-related synaptic plasticity in OFC or are they of foremost importance in acute information processing due to their slow-EPSP contributions? We found that the firing discrimination score increased significantly with learning during S+ and S- odor sampling in control sessions (Fig. 4.4A-B). On both S+ and S- trials, electrophysiological discrimination scores diverged between control and drug sessions with progressive learning, supporting the idea that learning-related plasticity of OFC firing patterns is reduced or lost with D-AP5 perfusion. Because the difference in discrimination scores between control and NMDAR blockade increased as learning progressed, the results suggest that OFC NMDARs are important for expressing long-term plasticity as underlying stimulus-outcome associative learning. Although our results do not prove that NMDA receptors mediate synaptic modifications within the OFC itself (because in theory they could also relay information acquired in afferent regions such as BLA; Groenewegen and Uylings, 2000; Mulder et al., 2003; Schoenbaum et al., 2003b), there are several indications that a mere relaying role can be considered unlikely. First, the NMDAR-mediated component of synaptic potentials in PFC is especially strong for recurrent, intracortical connections, not for excitatory inputs from afferent regions (Rotaru et al., 2011). Second, D-AP5 primarily affected OFC encoding during the cue period, and much less so during the later trial periods of movement, waiting and outcome. A crucial difference between the pre-decisional cue period and the post-decisional phases is that encoding of future outcome in these later periods does not necessarily depend on novel learning in the session under study, because it can rely on action patterns, cage- and well-related cues that have
already been associated with the outcome in previous learning sessions. If OFC NMDARs would merely relay previously acquired information from afferent regions, a stronger D-AP5 effect would have been expected also for these later trial periods. Nevertheless, this issue merits further investigation.

Regardless of the precise locus of plasticity, the question arises how NMDARs may support computational operations underlying decision-making involving OFC. In addition to the implication of OFC NMDARs in decision-making under reversal conditions (Bohn et al., 2003b), NMDARs in rat medial PFC affect appetitive instrumental learning (Baldwin et al., 2000). During odor discrimination learning, olfactory inputs need to be discriminated, and should be associated with outcome value as signaled later in the trial. After initial learning, cue value must be associatively recalled and coupled to an appropriate behavioral decision. Before the decision is executed, however, cue and value information may need to be retained in working memory. While NMDARs could in principle contribute to all of these operations, a few possibilities stand out.

Pattern discrimination, perceptual decision-making and maintenance in working memory have been proposed to be mediated by recurrent neural networks (Fig. 4.7; Lisman et al., 1998; Wang, 1999, 2002; Wong and Wang, 2006). In models of such networks, NMDARs on synapses between pyramidal
cells contribute to reverberating, sustained activity capable of slow integration of sensory evidence over time. Recent studies showed that NMDARs at pyramidal-pyramidal synapses in the deep layers of rat prefrontal cortex mediate sustained depolarization, that sustained synaptic activity recorded \textit{in vivo} from prelimbic cortex of anesthetized rats depended on NMDAR activity and that performance of a delayed-non-matching to sample task was impaired by NMDAR antagonists in dorsal hippocampus (Seamans et al., 2003; McHugh et al., 2008; Wang et al., 2008). Although such discriminatory and temporally-integrating mechanisms are predicted to operate during both early and late learning, the use and loading of recurrent network capacities may well change as learning progresses. In addition, OFC NMDARs may function in the actual updating of synaptic matrices encoding cue-outcome associations when reward contingencies are changing (Fig. 4.8; cf. Bohn et al., 2003b).

\textbf{Effect of NMDA receptor blockade on rhythmic synchronization}

Rhythmic synchronization, i.e. coupling of oscillatory activity across neurons and populations, has been hypothesized to play a role in the temporal coordination of neuronal activity between separate brain areas (Fries, 2009; Battaglia et al., 2011). Previous studies showed increments in gamma-band coherence in the hippocampus and frontal areas of awake rodents after peripheral application of non-competitive NMDAR antagonists (Ma and Leung, 2007; Pinault and Didier, 2008). Using a competitive NMDAR blocker, we did not observe a significant difference in gamma-band spike-LFP phase-locking between control and drug sessions, and found that NMDAR blockade did not abolish changes in LFP gamma power over trials (Fig. 4.5, 4.12). Consistent with this discrepancy, an \textit{in vitro} slice study showed that NMDAR blocking effects on gamma-band oscillations are highly dependent on the brain region under scrutiny and the mechanisms underlying gamma rhythmogenesis (Roopun et al., 2008). Slice studies further showed that NMDAR blockade increased the power of beta-band LFP oscillations in some areas (e.g. prelimbic and entorhinal cortex), but not in others (Middleton et al., 2008; Roopun et al., 2008). Thus, the emergence of a 20-25 Hz rhythm under a competitive NMDAR antagonist in behaving rats (Fig. 4.5C) may likewise be regionally specific. The occurrence of phase-locking to high-frequency (supra-gamma) oscillations with NMDAR blockade is consistent with a
similar, ketamine-induced increase observed in high-frequency oscillations in the striatum of awake rats (Hunt et al., 2011).

Several recent studies indicated that firing-rate selectivity can be predicted from a neuron’s pattern of synchronization to the LFP (Battaglia et al., 2011; Dean et al., 2012; Womelsdorf et al., 2012), suggesting that shared frequency and phase-of-firing preferences are a mechanism of neuronal assembly formation (Singer, 1999; Fries, 2005; Buzsáki, 2010). Here, we made a similar observation for the OFC: Neuronal firing rates were particularly selective to S+/S- conditions when their spiking activity was synchronized to the LFP theta rhythm (Fig. 4.6). NMDAR blockade abolished this relationship (Fig. 4.6) and reduced theta power over trials (Fig. 4.12). In addition, it caused firing rates to become less odor/outcome-selective when spikes were synchronized to supra-gamma frequencies. Together, these findings suggest a role for OFC NMDARs not only in firing rate odor selectivity but also in rhythmic synchronization as a mechanism to support this selectivity.
Chapter 4. NMDA-R IN OFC PLASTICITY

Figure 4.10 Stability of discrimination scores ($D_{\text{corrected}}$) from acquisition to reversal phase transition

(A) Pre-reversal (acquisition) $D_{\text{corrected}}$ values (Eq 2, mean ± SEM), an ROC-based measure of discrimination between S+ and S- trials, for the odor, early and late movement and waiting periods. For this particular analysis, only units were selected for which $D_{\text{corrected}}>0$. Note that average $D_{\text{corrected}}$ values are higher than in main Fig. 3, because for this particular analysis we selected only cells for which the inequality $D_{\text{corrected}}>0$ held true. (B) Same as (A), but now for post-reversal phase. (C) Post-reversal $D_{\text{corrected}}$ consistency values for all task periods. The $D_{\text{corrected}}$ consistency measure equaled the $[D_{\text{corrected}} \cdot C]$, where C was the selectivity consistency, defined as -1 if the firing rate selectivity for odor flipped from acquisition to reversal, and defined as +1 if the firing rate selectivity for odor was maintained from acquisition to reversal. *,**,*** of single groups: one-sample t-Test vs. 0, p<0.05,0.01,0.001 respectively. Underlined *,**,*** between groups: Mann-Whitney U-test, p<0.05,0.01,0.001 respectively.
Figure 4.11 Stability of discrimination scores \(D_{\text{raw}}\) from acquisition to reversal phase transition

(A) Pre-reversal (acquisition) \(D_{\text{raw}}\) values (Eq 1, mean ± SEM), an ROC-based measure of discrimination between S+ and S- trials, for the odor, early and late movement and waiting periods. For this particular analysis, only units were selected for which \(D_{\text{corrected}} > 0\). Note that average \(D_{\text{raw}}\) values are significantly higher than the \(D_{\text{corrected}}\) values in Fig. 4.10, because the \(D_{\text{raw}}\) measure is positively biased by the number of trials (since the shuffled value for \(D_{\text{corrected}}\) is not yet subtracted from \(D_{\text{raw}}\)). (B) Same as (A), but now for post-reversal phase. (C) Post-reversal \(D_{\text{raw}}\) consistency values for all task periods. The \(D_{\text{raw}}\) consistency measure equaled the \([D_{\text{raw}} \cdot C]\), where C was the selectivity consistency, defined as -1 if the firing rate selectivity for odor flipped from acquisition to reversal, and defined as +1 if the firing rate selectivity for odor was maintained from acquisition to reversal. Further plot conventions as in Fig. 4.10. Note that the Draw consistency significantly decreased relative to the Draw,rev values for all periods, except for the odor period in the control condition.
Chapter 4. NMDA-R IN OFC PLASTICITY

(continued from page 133)

As concerns NMDAR blockade effects in the odor phase, the Draw,rev consistency was significantly larger for aCSF than for D-AP5, was significantly decreased from the Draw,rev level in the drug, but not the control condition, and significantly exceeded zero only for the aCSF condition. Hence, D-AP5 distorts the maintenance of pre-reversal firing rate selectivities in the odor period in the reversal phase, suggesting that this phenomenon is an active process requiring support by this subtype of glutamate receptor.

Figure 4.12 Dependence of LFP power on trial number

Slopes of regression of average normalized LFP power onto trial number as a function of frequency. Shaded regions correspond to SEMs. Horizontal purple lines indicate significance at P<0.05 (Multiple comparison corrected Permutation test). Theta power decreased with trial number for the drug, but not control condition. The previously observed increase in gamma power with trial number (van Wingerden et al., 2010b) was not affected by NMDA-R blockade. The present findings on trial-dependent changes in LFP power increase go beyond Van Wingerden et al. (2010b) by showing that 1) the positive correlation between trial number and gamma power is confined to the gamma-frequency band (~60 Hz), and that 2) the spectrum of the correlation between power and trial number and power spectrum both have a band-limited peak in the same frequency-range (~60 Hz, cf. Fig. 4.5).
Table 4.1 Firing rate comparisons between aCSF and D-AP5 units

<table>
<thead>
<tr>
<th></th>
<th>Baseline (Hz)</th>
<th>Odor (Hz)</th>
<th>Move (Hz)</th>
<th>Wait (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw FR aCSF; 144 cells</td>
<td>2.35 ± 0.33</td>
<td>2.37 ± 0.38</td>
<td>2.56 ± 0.40</td>
<td>2.37 ± 0.39</td>
</tr>
<tr>
<td>Raw FR D-AP5; 110 cells</td>
<td>1.78 ± 0.32</td>
<td>2.36 ± 0.43</td>
<td>2.01 ± 0.28</td>
<td>1.96 ± 0.32</td>
</tr>
<tr>
<td>FR (% of base) aCSF</td>
<td>-</td>
<td>94 ± 4.7%</td>
<td>†</td>
<td>†</td>
</tr>
<tr>
<td>FR (% of base) D-AP5</td>
<td>-</td>
<td>136 ± 13%**</td>
<td>142 ± 21%</td>
<td>138 ± 10%***</td>
</tr>
</tbody>
</table>

Table 1 depicts the raw firing rate (FR) of putative pyramidal cells recorded under aCSF (N=144 of 164 aCSF cells) or D-AP5 (N=110 of 117 D-AP5 cells) conditions, as well as firing rates relative to baseline (set at 100%). Baseline was defined as -3 to -1 s before odor onset, the odor period (Odor) as 0 to +1 s after odor onset, the movement period (Move) as 0 to +1 s after odor offset and the waiting period (Wait) as -1 to 0 s before outcome delivery. Group averages ± SEM are reported. †Significant differences between group (aCSF, D-AP5) means, p<0.01 Mann-Whitney U-test. **,*** group mean significantly greater than baseline (100%), p<0.01, 0.001, one-sample t-Test versus 1).

Table 4.2 Distribution of firing-rate correlates across task periods

<table>
<thead>
<tr>
<th>Analyzed cells; N= 281</th>
<th>Odor</th>
<th>Move</th>
<th>Wait</th>
<th>Outcome</th>
<th>NONE</th>
</tr>
</thead>
<tbody>
<tr>
<td>aCSF sessions (N=10); N= 164 cells</td>
<td>29 (17.6%)</td>
<td>22 (13.4%)</td>
<td>21 (12.8%)</td>
<td>27 (16.5%)</td>
<td>99 (60.3%)</td>
</tr>
</tbody>
</table>

Distribution of cells recorded with a firing-rate change correlated to different task periods. Out of 281 analyzed cells, subsets responded to one or more of the following periods (such that the sum of percentages exceeds 100%). Odor: Odor sampling period; Move: Movement/locomotor period; Wait: waiting period; Fluid: fluid delivery period. Units in the ‘NONE’ category did not exhibit significant firing-rate correlates. Percentages in parentheses denote the fraction of cells out of the total number recorded under aCSF. Numbers in brackets indicate the (rounded) expected numbers of cells with correlates in D-AP5 sessions given the relative frequency of these correlates in aCSF sessions. The distribution of cell counts over the 5 categories did not significantly differ between aCSF and D-AP5 sessions (chi-square test, df = 4, p = 0.64).