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Nucleus Accumbens Core Acetylcholine is Preferentially Activated During Acquisition of Drug- *vs* Food-Reinforced Behavior

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Acquisition of drug-reinforced behavior is accompanied by a systematic increase of release of the neurotransmitter acetylcholine (ACh) rather than dopamine, the expected prime reward neurotransmitter candidate, in the nucleus accumbens core (AcbC), with activation of both muscarinic and nicotinic ACh receptors in the AcbC by ACh volume transmission being necessary for the drug conditioning. The present findings suggest that the AcbC ACh system is preferentially activated by drug reinforcers, because (1) acquisition of food-reinforced behavior was not paralleled by activation of ACh release in the AcbC whereas acquisition of morphine-reinforced behavior, like that of cocaine or remifentanil (tested previously), was, and because (2) local intra-AcbC administration of muscarinic or nicotinic ACh receptor antagonists (atropine or mecamylamine, respectively) did not block the acquisition of food-reinforced behavior whereas acquisition of drug-reinforced behavior had been blocked. Interestingly, the speed with which a drug of abuse distributed into the AcbC and was eliminated from the AcbC determined the size of the AcbC ACh signal, with the temporally more sharply delineated drug stimulus producing a more pronounced AcbC ACh signal. The present findings suggest that muscarinic and nicotinic ACh receptors in the AcbC are preferentially involved during reward conditioning for drugs of abuse *vs* sweetened condensed milk as a food reinforcer. *Neuropsychopharmacology* (2008) **33,** 3213–3220; doi:10.1038/npp.2008.48; published online 16 April 2008

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INTRODUCTION

Among the many brain areas involved in mediating the reinforcing effects of drugs of abuse and of physiological reinforcers such as food, the nucleus accumbens (Acb) holds a pivotal role (DiChiara and Imperato, 1988; Everitt *et al*, 2003; Koob, 2003). The Acb can be further subdivided into a 'shell' (AcbSh) region that evaluates (1) the interoceptive ('direct pharmacological') stimulus of the drug of abuse itself (Pontieri *et al*, 1995) and (2) drug-associated stimuli (discriminative stimuli as well as secondary reinforcing stimuli; Ciccocioppo *et al*, 2001; Ghitza *et al*, 2003; Ito *et al*, 2000) and a 'core' (AcbC) region that mediates the learned (conditioned) response to such stimuli, ie drug-seeking (Crespo *et al*, 2006; Ito *et al*, 2000; Koob *et al*, 2004; Neisewander *et al*, 2000; Peoples *et al*, 2004; Phillips *et al*, 2003; Shippenberg *et al*, 1992; Voorn *et al*, 2004). We recently demonstrated that acquisition of drug (ie cocaine (COC) or remifentanil, RMF)-reinforced behavior is accompanied by a systematic increase of release of the neurotransmitter acetylcholine (ACh) rather than dopamine (DA), the expected prime reward neurotransmitter candidate, in the AcbC (Crespo *et al*, 2006). Activation of both muscarinic (mAChR) and nicotinic (nAChR) acetylcholine receptors in the AcbC by ACh volume transmission was necessary for the drug conditioning (Crespo *et al*, 2006).

The present study was designed to investigate if the activation of AChRs in the AcbC during the acquisition of drug-reinforced behavior represents a general learning response or if it is preferentially engaged by drugs of abuse. To that end, we investigated the involvement of AcbC ACh activation during the conditioning to the physiological-reinforcer food (ie sweetened condensed milk) and compared it to AcbC ACh activation by morphine (MOR), a prototypical drug of abuse that belongs to the same pharmacological class as RMF, a μ-opioid receptor agonist, but is pharmacokinetically very different from RMF. MOR elimination from rat plasma is biphasic with an initial $t_{1/2}$ of 21 min (90% of total MOR; our own recalculation of the data obtained by Bhargava *et al* (1993a, b, c) and a terminal $t_{1/2}$ of 222 min (Bhargava *et al*, 1993b), whereas RMF's (monophasic) elimination half-life in rat blood/plasma ranges from 0.3 min (single dose; Crespo *et al*, 2005) to 0.69–0.75 min (postinfusion; Cox *et al*, 1999; Haidar *et al*, 1997). Unfortunately, comparative MOR-*vs*-RMF data on the distribution into brain areas are not available at a

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satisfactory temporal resolution. However, after i.v. administration, COC and RMF levels in the AcbC, as determined by *in vivo* microdialysis, peak in the first 10-min sample (Crespo *et al*, 2006), ie at 0–10 min, whereas AcbC MOR levels do not peak before the second 10-min sample, ie at 11–20 min (see below).

To summarize, the present study was designed (1) to compare the effects of a drug reinforcer *vs* a food reinforcer on AcbC ACh and DA release during acquisition of reinforced behavior in the rat runway operant conditioning paradigm, (2) to compare the effects of a local intra-AcbC administration mAChR and nAChR antagonists on conditioning of food *vs* drug reinforcement, and (3) to compare the effects on AcbC ACh and DA by two μ-opioid receptor agonist drug reinforcers which, although belonging to the same pharmacological class, dramatically differ with respect to their temporal definition as interoceptive stimulus at their CNS site of action, RMF being more sharply delineated temporally (fast distribution into the brain, fast elimination from the brain) than MOR (slower distribution, much slower elimination).

MATERIALS AND METHODS

Subjects and Drug Treatment

Male Sprague–Dawley rats were obtained from the Research Institute of Laboratory Animal Breeding (Himberg, Austria) weighing 250–300 g on receipt. Before surgery, all animals were housed in groups of six at a constant room temperature of 24°C and had free access to tap water. Rats were fed approximately 15.5 g (per day) pelleted chow (Tagger, Innsbruck, Austria), which kept them at about 90% of their free-feeding weight. All rats were tested during the light phase of a 12-h light–dark cycle (lights on at 0700 hours). The animals used in this study were cared for in accordance with the guidelines of the National Institutes of Health Animal Care and Use Program and the NIDA-IRP Animal Program, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Furthermore, the present experiments were approved by the National Animal Experiment Ethics Committee. MOR was obtained from the Innsbruck University Hospital Pharmacy, all other chemicals were obtained from Sigma-Aldrich (Vienna, Austria) unless indicated otherwise. Doses and concentrations refer to pure base.

Surgery

Male Sprague–Dawley rats were implanted under isoflurane (2–4%; Abbott, Vienna, Austria) anesthesia with concentric microdialysis probes (membrane: Filtral AN69; 40 000 molecular cutoff; 0.24 mm ID; 0.31 mm OD; Gambro Hospal, Wiener Neudorf, Austria) that had been assembled as detailed previously (Acquas *et al*, 2001; Taber *et al*, 1998; Zernig *et al*, 1997; Zernig and Fibiger, 1998). As unilateral (as opposed to bilateral) blockade of nAChRs or mAChRs in the AcbC by reverse microdialysis had proven sufficient to block the acquisition of drug-reinforced behavior (Crespo *et al*, 2006), in the present study, microdialysis probes were aimed at only the right-side AcbC region. The coordinates of the probe tip (with a 2 mm active dialysis membrane) relative to bregma were AP +1.6 mm, ML −1.6 mm, and DV 8.2 mm (Paxinos and Watson, 1998). Only experiments in which the probe tip location was confirmed by visual inspection of post-mortem brain slices to be within the AcbC limits as defined in the Paxinos and Watson atlas were included in the study.

Runway Procedure

In the operant runway procedure (Cohen and Ettenberg, 2007; Ettenberg, 2004; Geist and Ettenberg, 1990; Guzman and Ettenberg, 2007; McFarland and Ettenberg, 1998), the time that an animal needs to obtain a stimulus, the 'runtime', is thought to be inversely proportional to the reinforcing strength of that stimulus. Using this procedure, we could reliably demonstrate acquisition of drug-reinforced behavior for opioids and psychostimulants within only five consecutive trials in completely drug- and experiment-naive rats (Crespo *et al*, 2005, 2006; Wakonigg *et al*, 2003b). In the present study, 2 days after the implantation of the microdialysis probes, completely drug- and experiment-naive rats were given the opportunity to run for access to a food reinforcer, ie a 1 : 4 (v/v) dilution of sweetened condensed milk (Nestle, Vienna, Austria) for five consecutive trials ('runs'; inter-trial interval, 40 min) or for an i.v. injection of a drug reinforcer, ie MOR (1 mg/kg, i.v.). This MOR dose was chosen because it was the highest dose that proved to be a reliable positive reinforcer in previously published runway experiments from our group (see Figure 2 of Wakonigg *et al*, 2003b) and, therefore, was most likely to result in a robust *in vivo* microdialysis neurotransmitter release signal. The same criterion had previously (Crespo *et al*, 2006) caused us to choose an i.v. RMF dose of 0.032 mg/kg (see Figure 3 of Wakonigg *et al*, 2003b). The intertrial intervals for the food reinforcer were chosen to render the data directly comparable to our own previously published microdialysis-operant conditioning data on drug (ie COC and RMF) reinforcers (Crespo *et al*, 2006). Intertrial intervals for the MOR trials had to be as long as 90 min to allow elimination of >75% of the drug from the AcbC between runs (see below) to avoid direct pharmacological effects of MOR (eg sedation). Runs were started by opening a sliding door separating a start area from the main alley (length, 1 m) of the runway and by indicating availability of food or drug with a white cue light in the goal area. The click of a photobeam and the blinking of the cue light indicated the successful completion of the operant response for the run-contingent food or drug infusion. Sweetened condensed milk was made available via a spout extending through the wall of the runway's goal area. Spout access was only 20 s to avoid satiation, but rats were allowed to remain in the runway for 5 min. MOR was administered i.v. over a period of 6 s and rats were allowed to remain in the runway for 5 min. At the level of grossly observable behavior, when a positive reinforcer is made available in the goal area, the rats immediately leave the start area, ie commit to approaching the reinforcer-associated goal area immediately, and approach the goal area without engaging in alternative behavior (Crespo *et al*, 2005, 2006; Wakonigg *et al*, 2003b). In contrast, both an increase in the latency to

leave the start area and an increase in alternative behavior in the runway alley are consistently observed at operant level (i.v. saline as compared to i.v. drugs, or water as compared to sweetened condensed milk diluted in water). Runway dimensions and experimental details have been published previously (Wakonigg *et al*, 2003a, b, 2004).

In Vivo Microdialysis

In vivo microdialysis was performed during the behavioral experiments. The analytical probes were perfused with a 1 mM sodium phosphate-buffered solution containing (in mM): 147 NaCl, 3 KCl, 1 $MgCl_2$, and 1.3 $CaCl_2$ at 2 µl/min. The dialysate was immediately mixed with an HCl-based stabilizing solution, collected in 10-min samples (void volume of the probe outlet, <0.2 µl; 20 µl dialysate + 5 µl 50 mM HCl), and stored at $-70^{\circ}C$ for offline quantification. In some experiments, 10 µM atropine or 100 µM mecamylamine was added to the phosphate-buffered solution to administer the compound into the AcbC by reverse microdialysis. These concentrations, 10-fold higher than those generally found in brain slice superfusion experiments, were chosen to compensate for the spatial and temporal limitations associated with drug administration by reverse microdialysis. DA and ACh were simultaneously quantified by tandem mass spectrometry. To that end, 25 µl 10 mM HCl-stabilized dialysate were directly injected into the HPLC/MS/MS instrumentation. Chromatographic separation was performed on a Waters Acquity HSS T3 column (1.8 µM particle size; 2.1 × 100 mm) with a mobile-phase linear gradient starting at 95% 5 mM formic acid and 5% acetonitrile at a flow rate of 0.25 ml/min and ending at 100% acetonitrile. The eluate was directly injected into a Micromass Quattro Ultima triple quadrupole spectrometer (Waters, Vienna, Austria). Chromatographic peaks were identified using the following mass transitions: DA, $154 > 137$ *m/z*; ACh, $146 > 87$ *m/z*, and MOR, $286 > 165$ *m/z*. Details of the mass spectrometric detection of ACh and DA have been published previously (Crespo *et al*, 2006).

Data Analysis

Unless indicated otherwise, values are given as mean ± SEM of *N* determinations. Because of an experimenter-imposed cutoff of 60 s, group data for runtimes were first compared by the nonparametric repeated-measures corrected Friedman test followed by Dunn's multiple comparison test. All other group data were subjected to repeated-measures corrected two-factor (time, treatment) ANOVA followed by Tukey's multiple comparison test. The correlation of runtimes with ACh or DA peaks was investigated using Pearson's rank correlation test. Statistical tests were performed with Prism version 4 (GraphPad Software, San Diego, CA) or SPSS version 12.

RESULTS

Runtimes continuously and significantly decreased over the first five trials of the experiment if (1) the food reinforcer sweetened condensed milk (Figure 1, top) or (2) an i.v. injection of MOR (1 mg/kg; Figure 2, top) was made available in the goal area of the runway. This indicates that

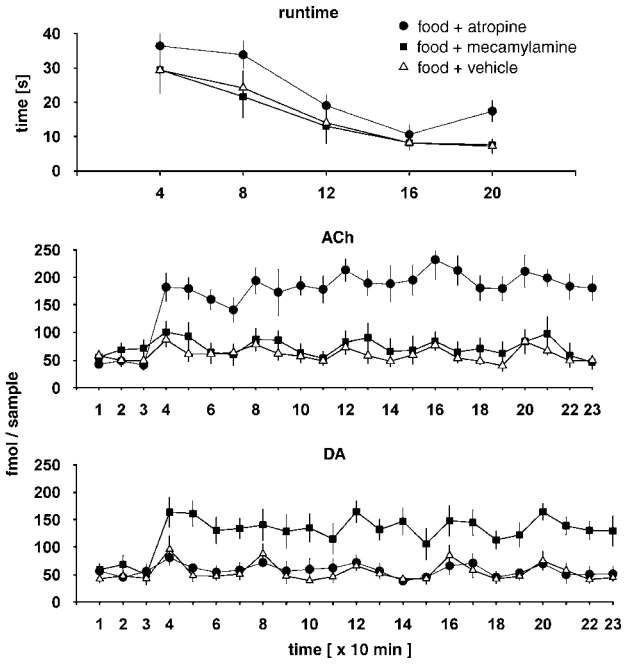

Figure 1 Acquisition of operant responding for food is not blocked by local intra-nucleus accumbens core (AcbC) muscarinic (m) or nicotinic (n) acetylcholine receptors (AChR) antagonists. Rats repeatedly ran for sweetened condensed milk in absence of any antagonist (vehicle control, $N = 6$, open triangles), in the presence of the mAChR antagonist atropine (10 µM; $N = 5$, filled circles), or in the presence of nAChR antagonist mecamylamine (100 µM; $N = 5$, filled squares), applied locally into the AcbC by reverse microdialysis (top, mean runtimes ± SEM). Behavior-associated overflow of ACh (center) and dopamine (DA, bottom) was assessed by simultaneous *in vivo* microdialysis. Consecutive number of 10-min samples, ie time (*x* axis); fmol per 10-min sample (mean ± SEM; *y* axis). Sample 4, run 1; 8, run 2; 12, run 3; 16, run 4; and sample 20, run 5. Baseline overflow (samples 1–3) of ACh and DA did not differ significantly between groups.

both the food stimulus and the i.v. MOR stimulus had acquired positively reinforcing properties. Two-factor ANOVA of runtimes in the food experiment (Figure 1) gave significance for time ($F(4, 52) = 17.22$; $p < 0.0001$) and treatment ($F(2, 52) = 5.742$; $p = 0.016$) but not for the time × treatment interaction ($F(8, 52) = 0.2408$; $p = 0.98$). With respect to the different treatments, however, *post hoc* multiple comparison (ie Bonferroni)-corrected statistical analysis revealed no significant difference between the food + vehicle group and the food + atropine group ($p > 0.05$) or between the food-vehicle group and the food + mecamylamine group ($p > 0.05$). ANOVA of runtimes for i.v. MOR (Figure 2) yielded an $F(9, 63) = 5.483$ ($p < 0.0001$).

If, in contrast to the reinforcer-induced systematic decreases in runtimes described in the present study, only water was made available to rats that were not water-deprived (ie operant level for food; Wakonigg *et al*, 2003b) or if only i.v. injections of saline were administered (ie operant level for i.v. drugs; Crespo *et al*, 2006; Wakonigg *et al*, 2003b) in the goal area, runtimes continuously increased to the experimenter-determined cutoff of 60 s (not shown in the present study).

Although runway behavior was remarkably similar between the drug and the food reinforcer, the AcbC ACh release pattern was strikingly different (Figures 1 and 2):

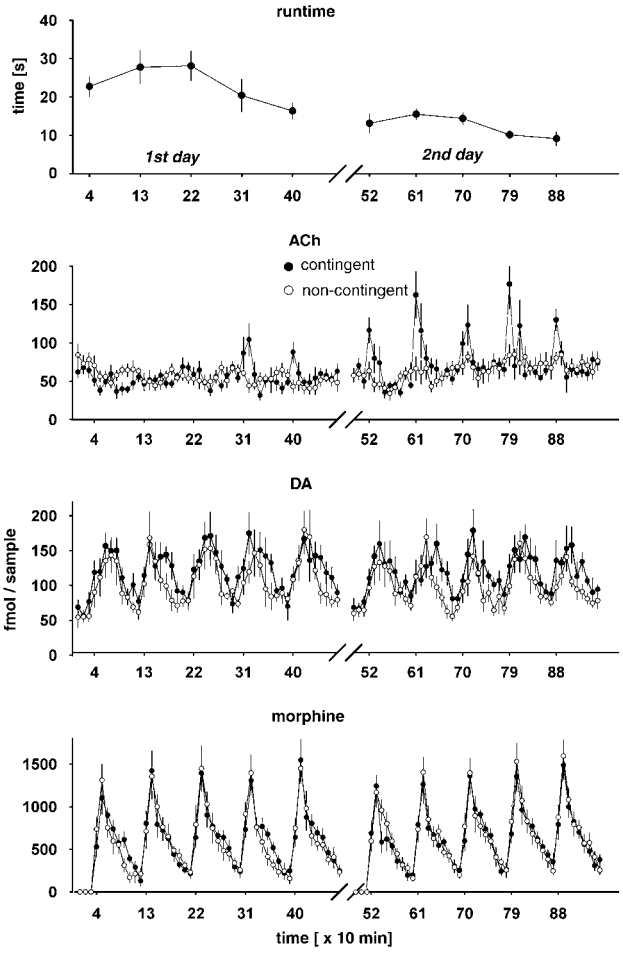

Figure 2 Acquisition of operant responding for morphine (MOR) is paralleled by a systematic increase in nucleus accumbens core (AcbC) acetylcholine (Ach) overflow. Sprague–Dawley rats were given the opportunity to run for an i.v. injection of 1 mg/kg MOR (contingent MOR) in a total of 10 trials distributed over 2 consecutive days. The top panel shows mean runtimes ± SEM ($N = 8$; first day: sample 4, run 1; 13, run 2; 31, run 4; 40, run 5; second day: sample 52, run 6; 61, run 7; 70, run 8; 79, run 9; and 88, run 10). Intertrial interval was 90 min. Another group of rats ($N = 6$) passively received the i.v. MOR injection within the confines of the runway (noncontingent MOR). Behavior-associated overflow of ACh (upper center panel) and DA (lower center panel) as well as intra-AcbC MOR levels (bottom panel) were assessed by simultaneous *in vivo* microdialysis. Consecutive number of 10-min samples, ie time (*x* axis); fmol per 10-min sample (mean ± SEM; *y* axis). One-factor (ie time) repeated-measures corrected ANOVA *p*-values were <0.0001 for runtimes and <0.0001 for contingent ACh peaks (see text). Repeated-comparison corrected *post hoc* analysis of the runtimes gave *p*-values of <0.05 for run 9 (sample 79) *vs* run 1 (sample 4) or for run 10 (sample 88) *vs* run 1 and, of the contingent ACh peaks, a $p<0.01$ for run 1 *vs* 7 (sample 61) and for run 1 *vs* 9 and a $p<0.05$ for run 1 *vs* 10.

response-contingent (ie self-administered) MOR, like the previously tested drug reinforcers RMF and COC (Crespo *et al*, 2006), produced a systematic and pronounced increase in AcbC ACh overflow upon repeated conditioning trials (to a maximum of 177 fmol per 10-min sample in run 9; Figure 2, center), whereas experimenter-delivered (ie noncontingent) MOR transiently increased AcbC ACh without producing a systematic, run-by-run increase in AcbC ACh peaks (Figure 2). The respective F-values were $F(9, 63) = 7.057$ ($p<0.0001$) for contingent MOR and $F(9, 45) = 1.797$ ($p = 0.0954$) for noncontingent MOR.

In contrast to MOR, food-associated AcbC ACh overflow remained near baseline (around 50 fmol per 10-min sample) across all five conditioning trials, showing run-associated peaks which rose only around 50% above baseline (to around 80 fmol per 10-min sample) and which did not change systematically or significantly over the five runs (Figure 1, center). Two-factor ANOVA gave the following F-values: $F(4, 52) = 0.1368$; $p = 0.9679$, for ACh peaks; $F(2, 52) = 72.45$; $p<0.0001$, for treatment; and $F(8, 52) = 0.4819$; $p = 0.8635$, for ACh peaks × treatment interaction. Intra-AcbC atropine, as expected and previously demonstrated and discussed (Crespo *et al*, 2006), led to a massive increase in AcbC ACh release (Figure 1, middle) without, however, significantly affecting runtimes (Figure 1, top) for food.

In contrast to the MOR-induced systematic changes in AcbC ACh and in confirmation of data previously obtained with COC and RMF (Crespo *et al*, 2006), MOR-induced AcbC DA peaks did not show any systematic change during subsequent runs (Figure 2, bottom; $F(9, 63) = 0.4532$; $p = 0.900$ for contingent and $F(9, 45) = 1.175$; $p = 0.3338$ for noncontingent MOR). The same held true for food-induced AcbC DA (Figure 1, bottom). Two-factor ANOVA gave the following F-values: $F(4, 52) = 0.4096$; $p = 0.8009$, for DA peaks; $F(2, 52) = 12.28$; $p = 0.0010$, for treatment; and $F(8, 52) = 0.3996$; $p = 0.9157$, for DA peaks × treatment interaction.

Also in contrast to drug reinforcers (Crespo *et al*, 2006), conditioning to the positive reinforcing effect of food could not be prevented by local intra-AcbC administration of either the mAChR antagonist atropine (10 μM; Figure 1, top) or the nAChR antagonist mecamylamine (100 μM; Figure 1, top). As expected from its inhibition of M2 mAChR autoreceptors, atropine considerably increased baseline AcbC ACh overflow, while at the same time abolishing all run-associated ACh peaks (Figure 1, center). In contrast, local intra-AcbC mecamylamine did not elevate overall AcbC ACh overflow but held it at baseline throughout the experiment. Finally, AcbC DA overflow also remained at baseline throughout the experiment if atropine was administered by reverse microdialysis (Figure 1, bottom). Intra-AcbC mecamylamine elevated basal AcbC DA overflow eliminating all run-associated DA peaks (Figure 1, bottom).

DISCUSSION

During the acquisition of its reinforcing effect, the physiological reinforcer food, ie sweetened condensed milk, engendered rat runway behavior that was remarkably similar to that produced by the drug reinforcer MOR (present study) and, as previously shown, RMF and COC (Crespo *et al*, 2006). In striking contrast to the drug reinforcers, however, the acquisition of food-reinforced behavior (1) was not paralleled by a systematic increase in AcbC ACh overflow and (2) could not be blocked by local intra-AcbC administration of a mAChR or nAChR antagonist. Thus, the present findings suggest that activation of the AcbC ACh system, which has been shown

to be necessary for the acquisition of drug-reinforced behavior (Crespo *et al*, 2006), may not be equally engaged in the conditioning to positive reinforcers in general, but may show a preference for drug *vs* food reinforcers. In general, striatal cholinergic interneurons are thought to be important in the formation of stimulus–response associations, ie learning (Aosaki *et al*, 1994; Calabresi *et al*, 2000; Kitabatake *et al*, 2003; Mansvelder *et al*, 2005; Suzuki *et al*, 2001). This also seems to hold true for the AcbC, a striatal structure dedicated to processing stimuli of high emotional and motivational valence (Haber *et al*, 1985; Jongen-Relo *et al*, 1994). In modification of the above rule, the present study suggests that learning-associated ACh interneuron activity in the AcbC might be preferentially involved during the conditioning to drugs of abuse rather than to positively reinforcing stimuli, such as food, in general. Our findings are in good agreement with data by Smith *et al* (2004) who showed that neurotoxic ablation of cholinergic interneurons in the posterior Acb and ventral pallidum by 192-IgG-saporin flattened the dose–response curve for COC self-administration although essentially leaving food self-administration intact. Of note, some would argue that the Acb 192-IgG-saporin lesion by Smith *et al* may have targeted not Acb ACh interneurons but cholinergic projection neurons from the ventral pallidum and other nearby basal forebrain structures as well (see discussion in Smith *et al*, 2004). Our findings on the cholinergic modulation of drug reinforcement are also in good agreement with recent findings by Mark and colleagues who showed that systemic (i.v.) mecamylamine prevented escalation of COC self-administration (Hansen and Mark, 2007) in the long-access (ie 6 h) paradigm developed by Ahmed and Koob (1998) (see also Lenoir and Ahmed, 2007).

At first sight, our findings are at variance with the work by Pratt and Kelley (2004) who showed (1) that blockade of mAChR in the AcbC or AcbSh prevented the acquisition of lever press responding for sucrose, and that intra-AcbC infusion of the mAChR antagonist scopolamine (2) decreased the salience of sucrose-associated contextual or visual stimuli (Pratt *et al*, 2007), and (3) decreased 24 h food intake (Pratt and Kelley, 2005). A possible explanation for this variance is the fact that in our operant conditioning paradigm of food responding, the muscarinic or nicotinic antagonist was administered only unilaterally, whereas Pratt and Kelley employed bilateral injections, suggesting that the lack of modulation of food responding by unilateral intra-AcbC blockade of mAChRs or nAChRs as reported in the present study, although being sufficient to completely block responding for drugs of abuse (Crespo *et al*, 2006) may indicate a graded, preferential modulation of drug over food responding by intra-AcbC mAChRs and nAChRs and that blockade of AcbC mAChRs in both hemispheres of the brain—a rather drastic pharmacological intervention—is necessary to eliminate responding for both food and drugs of abuse. As a further experimental difference, Pratt and Kelley (2005) administered the antagonists repeatedly for very brief (ie 2.33 min) periods of time through cannulae, whereas in the present experiments, the antagonists were continuously infused via reverse microdialysis for over 3 h, but only during a single experimental session. Our findings, ie the lack of an effect on food-reinforced responding by intra-AcbC mecamylamine or atropine, are also at odds with the work by Hoebel *et al* (2007) who have demonstrated that ACh release in the Acb is a correlate of meal satiation, conditioned taste aversion, and aversive brain stimulation. Currently, we have no explanation for this discrepancy.

The facts that the AcbC cholinergic system (1) is activated in drug reinforcement (Crespo *et al*, 2006; Mark *et al*, 1999; present study) and that (2) local lesioning of cholinergic interneurons in the Acb (Smith *et al*, 2004) lead to a progressive loss of the reinforcing effect of COC over the course of 24 days (of Smith *et al*, 2004) seems at odds with a number of experiments demonstrating that inhibition rather than activation of the cholinergic system enhances apparent (Zernig *et al*, 2007) drug reinforcement (Hikida *et al*, 2001, 2003; Wilson and Schuster, 1973). We propose that this apparent discrepancy can be resolved if one assumes that the involvement of the (accumbal) cholinergic system is important during the acquisition of drug-reinforced behavior, whereas the cholinergic system is of much less importance once drug-taking has become habitual (Everitt and Robbins, 2005), which can easily be imagined to be the case in self-administering animals with a long history of drug training (Zernig *et al*, 2007). We propose that during habitual drug-taking, activation of the cholinergic system may actually increase the attention of the drug-taking individual toward alternative reinforcers, thus in effect inhibiting habitual drug-taking.

In the course of the present investigation, we also found that the speed of drug conditioning (ie learning) and the size and pattern of change of the AcbC ACh signal associated with this learning process depended on the drug's temporal definition as an interoceptive stimulus at its CNS site of action: RMF, albeit from the same pharmacological class (ie μ-opioid receptor agonists) as MOR, presents a temporally much better defined stimulus than MOR because of its faster distribution into the AcbC as a deep brain area (RMF, 0–10 min; Crespo *et al*, 2006 *vs* MOR, 11–20 min; Figure 2) and because of its much faster elimination from the brain (RMF, 0.3–0.75 min; Crespo *et al*, 2005 *vs* MOR, 21–222 min; Bhargava *et al*, 1993b). Of note, COC, with its distribution into the AcbC between 0 and 10 min and elimination $t_{1/2}$ of 10 min from the AcbC (Crespo *et al*, 2006) is pharmacokinetically very similar to RMF (see Zernig *et al*, 2007 for a detailed discussion), although belonging to a different pharmacological class (psychostimulants). Accordingly, rats acquired the runway operant response for RMF or COC within 3–5 trials (see Figures 1 and 2 of Crespo *et al*, 2006), whereas it took the rats at least five trials (significant differences from baseline only at trials 9 and 10) to acquire the operant response for MOR (Figure 2, top). Furthermore, although the baseline AcbC ACh was identical for all three drug reinforcers (ie around 50 fmol per 10-min sample), the learning-associated systematic increase in AcbC ACh peaks was much more pronounced for RMF (1243 ± 489 fmol per 10-min sample) and COC (408 ± 79 fmol per 10-min sample) (Crespo *et al*, 2006) than for MOR (177 ± 32 fmol per 10-min sample; Figure 2, center; $p < 0.001$ with respect to either COC or RMF). It, thus, seems that the better temporal definition (ie more rapid onset, more rapid offset) of the RMF- or COC-stimulus facilitated the acquisition of the operant response (ie learning) for the drug stimulus and led to a

much more pronounced learning-associated systematic increase in the AcbC ACh signal. Again, these AcbC ACh changes seem to be selective for drug reinforcers as the food stimulus, albeit also displaying a very distinctive rapid onset (ie food presentation by spout in the goal area) and distinctive rapid termination (ie spout removed after 20 s and animal removed from the runway by the experimenter after 5 min) enabled fast learning (complete within 3–5 trials; Figure 1, top) but failed to change the Acb ACh signal in any systematic way (Figure 1, center). Thus, the excellent temporal definition of the reinforcing stimulus engendered more rapid learning (ie the formation of response–stimulus associations; Zernig *et al*, 2007) despite the fact that the two investigated reinforcers, ie the drug-reinforcer RMF and the food-reinforcer sweetened condensed milk, were qualitatively very dissimilar. However, despite their similarity with respect to the speed of conditioning, the food reinforcer failed to affect AcbC ACh release (present study) whereas the drug-reinforcer RMF (Crespo *et al*, 2006)—or MOR, a drug reinforcer from the same pharmacological class that engendered conditioning at a lower speed than either food or RMF—did produce a systematic increase in AcbC ACh release.

The absence of a systematic (ie run 1 *vs* 5) increase in AcbC DA peaks upon response-contingent presentation of MOR and food in the present study is at odds with findings obtained during the conditioning of a food-associated olfactory stimulus (Bassareo and DiChiara, 1999) in which a single trial was sufficient to produce stimulation of AcbC DA by the cheese-like smell of a snack food in a subsequent trial 5 days later. Maybe differences in the experimental protocol (eg olfactory *vs* visual conditioned stimulus, intertrial intervals of 40–90 min in the present study *vs* 5 days in the study by Bassareo and DiChiara, 1999) account for this discrepancy. As had been the case for COC and RMF (Crespo *et al*, 2006), ie conforming to drugs of abuse in general, noncontingent MOR also increased AcbC ACh and AcbC DA (Figure 1, middle). For a further discussion of these phenomena, the reader is referred to a previous publication (Crespo *et al*, 2006).

The absence of any changes in AcbC ACh seen in the present *in vivo* microdialysis study might miss a possible important contribution of fast cholinergic transients to the conditioning of food reinforcement which might be demonstrated with the help of experimental approaches that offer higher temporal and spatial resolution, eg electrophysiological tests. Our experimental approach is also unable to capture any fast ACh-mediated changes in its signal transduction cascade.

One might argue that the different cholinergic response to a drug- *vs* a food reinforcer might be a general time-related phenomenon, rather than being strictly governed by the nature of the appetitive stimulus (food *vs* drug of abuse). However, as the same intertrial interval of 40 min was used for two drug reinforcers from different pharmacological classes, ie COC and RMF (Crespo *et al*, 2006), and for food (Figure 1), producing a systematic increase in AcbC ACh for the drug reinforcers but not the food reinforcer, we think that the cholinergic response is not a general time-related phenomenon.

Another argument for a general learning-related phenomenon would posit that had we extended the experiments with the food reinforcer to 10 trials as we did with the MOR reinforcer we might have observed a similar change in the AcbC ACh signal. There is, however, no indication for any systematic increase in AcbC ACh release in the contingent food experiment (Figure 1) in the first five trials, whereas contingent MOR produced a pronounced increase in AcbC ACh release as early as in trial 4 (Figure 2). In addition, two other drugs of abuse, ie COC and RMF, had produced a massive systemic increase in the AcbC ACh signal well within the first five trials (Crespo *et al*, 2006), ie at a time when there was no indication that the acquisition of food-reinforced behavior produced any systematic change in AcbC ACh. To summarize, although we cannot exclude the possibility that food reinforcement could eventually also lead to an increase in the AcbC ACh signal, it can safely be assumed that drug reinforcers preferentially activate the AcbC cholinergic system.

Finally, traversing a rat runway alley to obtain a reinforcer at its end might constitute less of a *bona fide* operant (specifically aimed at obtaining the reinforcer) than simple Pavlovian approach (a more general behavior that is elicited by drug stimuli and, more importantly, by drug-associated stimuli; for a detailed discussion see Zernig *et al*, 2007). Thus, the operant runway paradigm, originally developed and continuously refined by Ettenberg and co-workers (Cohen and Ettenberg, 2007; Geist and Ettenberg, 1990, 1997) might assess more the Pavlovian incentive value (also called 'incentive salience') of drug-associated stimuli (sometimes called drug 'wanting') than the goal status or incentive value of the food or drug stimulus (Zernig *et al*, 2007). Despite this shortcoming, approach behavior does have excellent face value when assessing the attractiveness of a reinforcer (Zernig *et al*, 2007).

In conclusion, the present findings suggest that mAChR and nACh in the nucleus AcbC are preferentially involved during reward conditioning for drugs of abuse *vs* sweetened condensed milk as a food reinforcer.

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Dr Zernig has received compensations as a consultant for the following companies (in alphabetical order), which are manufacturers of antidepressants, antipsychotics, analgesics, hypnotics, or opioid substitution medications: Janssen, Kwizda, Lundbeck, Mundipharma, Nycomed, and Pfizer. He has held compensated yet unrestricted lectures on problems of therapeutic drug monitoring (TDM) for (in alphabetical order): AstraZeneca, Bristol-Myers Squibb, Eli Lilly, Glaxo-SmithKline, Janssen, Lundbeck, and Pfizer. Dr Zernig has no personal financial holdings that could be perceived as constituting a potential conflict of interest.

All other authors declare that except for income received from their primary employer no financial support or compensation has been received from any individual or

corporate identity over the past 3 years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

All authors declare that they have no conflict of interest with respect to the contents of the present study.

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