Effects of MDMA on the pupillary light reflex on its own and after pretreatment with reboxetine, duloxetine, clonidine, carvedilol, and doxazosin

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Running title: MDMA and pupillary function

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Abstract

Rationale: Pupillometry can be used to characterize autonomic drug effects. *Objective:* To determine the autonomic effects of 3,4-methylenedioxymethamphetamine (MDMA, ecstasy) on pupillary function, administered alone and after pretreatment with reboxetine, duloxetine, clonidine, carvedilol, and doxazosin. *Methods:* Infrared pupillometry was performed in five placebo-controlled randomized clinical studies. Each study included 16 healthy subjects (eight men, eight women) who received placebo-MDMA (125 mg), placebo-placebo, pretreatment-placebo, or pretreatment-MDMA using a crossover design. *Results:* MDMA produced mydriasis, reduced the response to light, prolonged the latency to the light reflex, and shortened the recovery time. The impaired reflex response was short-lasting and associated with subjective, cardiostimulant, and hyperthermic drug effects and returned to normal within 5-6 h after MDMA administration when plasma MDMA levels were still high. Mydriasis was associated with the changes in plasma MDMA concentration over time and longer-lasting. Both reboxetine and duloxetine interacted with the effects of MDMA on pupillary function. Duloxetine even prevented MDMA-induced impairments in the light reflex response despite having similar effects when administered alone. Clonidine did not significantly reduce the mydriatic effects of MDMA, although it produced miosis when administered alone. Carvedilol and doxazosin did not alter the effects of MDMA on pupillary function. *Conclusions:* The MDMA-induced prolongation of the latency in the pupillary light reflex response and the reduction of light-induced miosis indicate indirect central parasympathetic inhibition, and the faster recovery time reflects an increased direct sympathomimetic action. Both norepinephrine and serotonin mediate the effects of MDMA on pupillary function. While MDMA-induced mydriasis is lasting and mirrors the plasma concentration-time curve of MDMA, the impairment in the reaction to light is associated with the subjective and other autonomic effects induced by MDMA and exhibits acute tolerance.

Keywords: Pupil, pupillary reflex, pupillometry, MDMA, norepinephrine, serotonin

Introduction

3,4-Methylenedioxymethamphetamine (MDMA, ecstasy) induces the transporter-mediated release of serotonin and norepinephrine (Liechti and Vollenweider 2001; Rothman et al. 2001; Verrico et al. 2007) and produces cardio- and psychostimulant effects in humans (Hysek et al. 2011). The autonomic sympathomimetic effects of MDMA in humans include increases in blood pressure, heart rate, body temperature, and pupil diameter (Farre et al. 2004, 2007; Hysek et al. 2012c; Kolbrich et al. 2008; Liechti et al. 2001; Mas et al. 1999). Pupil size and the response to a flashlight stimulus are typically assessed in the evaluation of intoxicated patients. Mydriasis is a clinical hallmark of sympathomimetic toxicity in cases of ecstasy or cocaine use. Laboratory studies have also shown an increase in pupil diameter after MDMA administration (Farre et al. 2004, 2007; Kolbrich et al. 2008; Mas et al. 1999). However, unknown are whether MDMA alters the pupillary light reflex response and how pupillary changes are linked to MDMA exposure and the subjective effects of the drug. Additionally, the pharmacological mechanism by which MDMA produces mydriasis and potential changes in pupillary function are unclear. Mydriasis and alterations in the pupillary light reflex may result from increased sympathetic activity, the release of norepinephrine, and α_1-adrenergic receptor stimulation directly in the iris or from a decrease in parasympathetic activity (Loewenfeld 1999). At the level of the iris, the latency to the light reflex and miotic response to light are thought to reflect parasympathetic activation (Heller et al. 1990; Loewenfeld 1999), whereas redilation is considered to mainly reflect sympathetic activation (Loewenfeld 1999; Morley et al. 1991). Notably, the parasympathetic input to the pupil may also be inhibited centrally via α_2-adrenergic receptors in the Edinger-Westphal nucleus by an increase in sympathetic activity (Phillips et al. 2000a; Siepmann et al. 2007; Szabadi and Bradshaw 1996). Furthermore, the serotonin system has been shown to indirectly influence pupillary function, possibly by enhancing sympathetic activity (Prow et al. 1996). Therefore, the MDMA-induced release of norepinephrine in the periphery may stimulate α_1-adrenergic receptors in the iris or inhibit parasympathetic activity via central α_2-adrenergic receptors in the Edinger-Westphal nucleus. The adrenergic mechanisms may be further enhanced by the

potent MDMA-induced release of serotonin. To explore the mechanism of action of MDMA on pupillary function, we investigated the effects of five pretreatments on the response to MDMA. We used the norepinephrine transporter inhibitor reboxetine to block the transporter-mediated, MDMA-induced release of norepinephrine (Hysek et al. 2011; i.e., the indirect sympathomimetic effect of MDMA). The serotonin and norepinephrine transporter inhibitor duloxetine was similarly used to block the MDMA-induced, transporter-mediated release of both serotonin and norepinephrine (Simmler et al. 2011). The α_2-adrenergic agonist clonidine was used as a sympathicolytic to inhibit the transporter-independent vesicular release of norepinephrine (Hysek et al. 2012a). Carvedilol and doxazosin were used to block postsynaptic $\alpha_1\beta_{1\text{-}3}$- and α_1-adrenergic receptors, respectively (Hysek et al. 2012c; i.e., to directly antagonize the effects of norepinephrine in the iris, on the cardiovascular system, and on body temperature). The large series of studies included additional outcome measures presented elsewhere (Hysek et al. 2011, 2012a, b, d; Simmler et al. 2011).

Material and Methods

Study design

This was a pooled analysis of five double-blind, double-dummy placebo-controlled, randomized, crossover studies (Hysek et al. 2011, 2012a, b, d; Simmler et al. 2011). The primary aim of the pooled analysis was to assess the effects of MDMA on pupil size and the pupillary light reflex compared with placebo in all 80 subjects and explore associations with the pharmacokinetics of MDMA and other pharmacodynamic measures. All of the subjects included in the five studies received MDMA, placebo, one of five different pretreatments prior to MDMA, or the pretreatment alone (Fig. 1). Thus, the four experiential conditions for all of the subjects were placebo-placebo, pretreatment-placebo, placebo-MDMA, and pretreatment-MDMA in a balanced order. Each of the five studies included 16 subjects (eight male, eight female). The pretreatments used in the five studies were reboxetine, duloxetine, clonidine, carvedilol, and doxazosin. The random allocation sequence was developed by a clinical pharmacist and concealed from all of the individuals involved in study management.

The washout periods between sessions were ≥ 10 days. The studies were conducted in accordance with the Declaration of Helsinki and International Conference on Harmonization Guidelines on Good Clinical Practice and approved by the Ethics Committee of the Canton of Basel, Switzerland. The use of MDMA in healthy subjects was authorized by the Swiss Federal Office of Public Health, Bern, Switzerland. The studies were registered at ClinicalTrials.gov (NCT00886886, NCT00990067, NCT01136278, NCT01270672, and NCT01386177).

Participants

Eighty healthy subjects (40 men and 40 women) aged 18 to 44 years (mean ± SD, 25 ± 5 years) were recruited on the university campus. The exclusion criteria included the following: (*i*) age < 18 or > 45 years, (*ii*) pregnancy determined by a urine test before each test session, (*iii*) body mass index < 18.5 kg/m^2 or > 25 kg/m^2, (*iv*) personal or family (first-degree relative) history of psychiatric disorder (determined by the structured clinical interview for Axis I and Axis II disorders according to the *Diagnostic and Statistical Manual of Mental Disorders*, 4th edition [Wittchen et al. 1997], supplemented by the SCL-90-R Symptom Checklist [Derogatis et al. 1976; Schmitz et al. 2000]), (*v*) the regular use of medications, (*vi*) chronic or acute physical illness assessed by physical examination, electrocardiogram, standard hematology, and chemical blood analyses, (*vii*) smoking more than 10 cigarettes per day, (*viii*) a lifetime history of using illicit drugs more than five times, with the exception of cannabis, (*ix*) illicit drug use within the last 2 months, and (*x*) illicit drug use during the study determined by urine tests conducted before the test sessions using TRIAGE 8 (Biosite, San Diego, CA). The subjects were asked to abstain from excessive alcohol consumption between test sessions and limit alcohol use to one drink on the day before each test session. Eight of the 80 subjects had previously tried ecstasy (one to two times). Female subjects were investigated during the follicular phase (day 2-14) of their menstrual cycle to account for the potential confounding effects of sex hormones and cyclic changes in the reactivity to

amphetamines (White et al. 2002). All of the subjects provided their written informed consent before participating in the study, and they were paid for their participation.

Measures

Pupillometry. Pupillometry was performed 1 h before and 0, 0.33, 0.66, 1, 1.5, 2, 2.5, 3, 4, 5, and 6 h after MDMA or placebo administration. Pupil function was measured under standardized dark-light conditions of 5.7 ± 0.8 lux assessed by a Voltcraft MS-1300 luxmeter (Voltcraft, Hirschau, Germany) following a dark adaption time of 1 min. Pupillometry was performed using a hand-held PRL-200 infrared pupillometer (NeurOptics, Irvine, CA; Taylor et al. 2003). The subjects were instructed to focus on a black dot on a white wall at a distance of 4 m. After a 10 s focusing period, measurements were taken for 5 s. During this time frame, the following parameters were assessed: dark-adapted pupil diameter (MAX), minimal pupil diameter after a light stimulus (MIN), and latency to the pupillary light reflex (Fig. 2). The constriction amplitude was calculated as MAX-MIN. The time taken by the pupil to recover 75% of the initial resting pupil size after it reached constriction was also assessed. The dynamic pupil measurements were triggered by a light impulse of 180 μW intensity and 167 ms duration. Measurements were performed on both eyes, and the average values were used for further analyses.

Subjective drug effect. Subjective drug effects were assessed using visual analog scales (VASs) reported in detail elsewhere (Hysek et al. 2011, 2012a). In the present report, we included only the VAS rating of “any subjective drug effects,” measured using a 100-mm horizontal line marked “not at all” on the left and “extremely” on the right. The VAS was repeatedly administered 1 h before and 0, 0.33, 0.66, 1, 1.5, 2, 2.5, 3, 4, 5, and 6 h after MDMA or placebo administration. The scale is very sensitive to the overall psychotropic effects of MDMA. The comprehensive assessment of different aspects of the psychotropic response to MDMA have been presented in the reports of the individual studies (Hysek et al. 2011, 2012a, 2012b, 2012d; Simmler et al. 2011).

Blood pressure, heart rate, and body temperature. Blood pressure and heart rate were assessed repeatedly 1h before and 0, 0.33, 0.66, 1, 1.5, 2, 2.5, 3, 4, 5, and 6 h after MDMA or placebo administration using an OMRON M7 monitor (Omron Healthcare Europe, Hoofddorp, The Netherlands) in the dominant arm and after a resting time of 5 min. Measures were taken twice per time point with an interval of 1 min, and the average was used for analysis. Mean arterial pressure (MAP) was calculated from diastolic and systolic blood pressure using the formula MAP = diastolic blood pressure + (systolic blood pressure – diastolic blood pressure)/3. Core (tympanic) temperature was assessed repeatedly in the same intervals as blood pressure and heart rate using a GENIUS 2 ear thermometer (Tyco Healthcare Group, Watertown, NY, USA).

Pharmacokinetics of MDMA. Blood samples were collected before and 0, 0.33, 0.66, 1, 1.5, 2, 2.5, 3, 4, and 6 h after MDMA or placebo administration, and plasma MDMA levels were determined as previously described (Hysek et al. 2012a). The data for the plasma concentrations of MDMA were analyzed using noncompartmental methods. Maximal plasma concentration and the time to maximal plasma concentration were obtained directly from the concentration-time curves of the observed values. Plasma concentrations were only determined up to 6 h after MDMA administration because the aim of the study was to assess plasma exposure only during the time of the pharmacodynamic effects of MDMA.

Drugs

(±) MDMA hydrochloride (Lipomed AG, Arlesheim, Switzerland) was prepared as gelatin capsules (100 and 25 mg). Identical placebo (mannitol) capsules were prepared. MDMA was administered in a single absolute oral dose of 125 mg. This dose of MDMA corresponds to a typical recreational dose or the dose of MDMA used as an adjunct to psychotherapy (Mithoefer et al. 2010). In the reboxetine-MDMA study, reboxetine (Edronax; 8 mg; Pfizer, Zurich, Switzerland) or identical placebo was administered at 8:00 PM on the day before the test session and again at 7:00 AM on the test day. MDMA or placebo was administered at 8:00 AM, 1 and 12 h after reboxetine. In the duloxetine-MDMA study,

duloxetine (Cymbalta; 120 mg; Eli Lilly, Vernier, Switzerland) or identical placebo was administered at 8:00 PM on the day before the test session and again at 8:00 AM on the test day. MDMA or placebo was administered at 12:00 PM, 4 and 16 h after duloxetine. Reboxetine and duloxetine were administered twice at high doses to obtain peak plasma concentrations of (mean ± SD) 372 ± 34 ng/ml and 107 ± 10 ng/ml, respectively, similar to the concentrations reached with chronic daily administration of 4 mg and 60 mg of the drugs, respectively (Hysek et al. 2011; Simmler et al. 2011), and as previously used to manipulate noradrenergic function in healthy subjects (Roelands et al. 2008). Compliance with the first administration of reboxetine and duloxetine on the evening prior to the test day was confirmed analytically in plasma (Hysek et al. 2011, 2012d). In the clonidine-MDMA study, clonidine (Catapresan; 150 µg; Boeringer Ingelheim, Basel, Switzerland) or identical placebo was administered at 8:00 AM, 1 h before MDMA or placebo (9:00 AM; Hysek et al. 2012a). Clonidine has previously been shown to produce sympatholytic effects at this dose in healthy subjects (Anavekar et al. 1982; Bitsios et al. 1996; Nieuwenhuis et al. 2007) and was expected to produce peak plasma concentrations in the range of 0.6-0.7 ng/ml (Anavekar et al. 1982; Keranen et al. 1978). In the carvedilol-MDMA study, carvedilol (Dilatrend; 50 mg; Roche, Basel, Switzerland) or identical placebo was administered at 8:00 AM, 1 h before MDMA or placebo (9:00 AM; Hysek et al. 2012c). The same dose of carvedilol has previously been shown to attenuate the smoked cocaine-induced increases in heart rate and blood pressure in humans (Sofuoglu et al. 2000) and was expected to produce peak plasma concentrations in the range of 120-180 mg/ml (Henderson et al. 2006; Morgan 1994). At this dose, carvedilol is expected to inhibit both α_1- and β-adrenergic receptors (Sofuoglu et al. 2000; Tham et al. 1995), with 5- to 10-fold higher activity at β-receptors (Tomlinson et al. 1988, 1992). In the doxazosin-MDMA study, continued-release doxazosin (Cardura; 4 mg; Pfizer, Zurich, Switzerland) or identical placebo was used. A first dose of 4 mg of doxazosin was administered 3 days before MDMA or placebo (-64 h) at 5:00 PM, a second dose of 8 mg was administered 2 days before MDMA or placebo (-40 h) at 5:00 PM, and a third dose of 8 mg was administered the day before MDMA or placebo administration (-16 h) at 5:00

PM. The subjects were reminded by a phone call or phone text message to ingest the capsules, and medication containers were checked to confirm that the first two doses of doxazosin were administered. The last administration was supervised by study personnel at the research facility. This administration schedule accounted for the long t_{max} of 8-10 h of the continuous-release formulation of doxazosin and reduced the risk of hypotension (Chung et al. 1999). Based on similar dosing regimes in healthy subjects (Chung et al. 1999; Shirai et al. 2010), the mean estimated peak plasma concentration of doxazosin was 30 $\pm$ 5 ng/ml, similar to the concentration with steady state-dosing of 4 mg (Chung et al. 1999). The pretreatment times for the administration of the five pretreatments resulted in maximal plasma concentrations of the pretreatments at the time of or shortly before the maximal effect of MDMA, based on our analytical results (Hysek et al. 2011; Hysek et al. 2012a, c, d) or published data (Anavekar et al. 1982; Chung et al. 1999; Henderson et al. 2006; Keranen et al. 1978; Morgan 1994; Shirai et al. 2010). Oral drug administration on the test days was supervised by study personnel.

Statistical analyses

Maximal effect values (E_{max}), minimal effect values (E_{min}; only for clonidine), and areas under the effect-time curves were determined with repeated measures. Values from the five studies were separately compared using two-way factorial General Linear Models repeated-measures analysis of variance (ANOVA), with the factors MDMA (MDMA *vs*. placebo) and pretreatment (pretreatment *vs*. placebo), using STATISTICA 6.0 software (StatSoft, Tulsa, OK). Additionally, MDMA and placebo values from all of the studies were pooled and analyzed with MDMA as a single within-subjects factor. Tukey *post hoc* comparisons were performed based on significant main effects or interactions in the ANOVA. Analyses of the area under the effect-time curve data yielded identical results to those of the maximal values and are therefore not shown. Associations between the pharmacodynamic changes and plasma concentration of MDMA were analyzed using Spearman's rank correlations. This first correlation analysis assessed associations of the parameters between

subjects (n = 80) for each time point. The mean pharmacodynamic changes after MDMA administration for each time point were then plotted against the respective mean plasma concentrations of MDMA and graphed as hysteresis curves. Correlations between the pharmacodynamic-pharmacokinetic data pairs over time (n = 10 time points) were then analyzed using Spearman's rank correlation. Associations between pupillary function parameters and subjective effects were similarly analyzed (n = 11 time points). This second correlation analysis assessed the associations of mean parameters over time within the 16 subjects (n = 9 or 10). The criterion for significance was $p < 0.05$

Results

Parameters of pupillary function (placebo condition)

Pupillary function parameters were measured 12 times throughout the day in 80 subjects. For placebomean ± SEM values were the following: pupil size = 6.23 ± 0.09 mm, pupil size after light = 4.34 ± 0.08 mm, constriction amplitude = 1.90 ± 0.01 mm, and recovery time 2.46 ± 0.06 s. Maximal values are shown in Table 1. The diameter of the light-stimulated pupil correlated with resting pupil size prior to the light stimulus (R_s =0.94, $p < 0.001$, n = 80).

Effects of MDMA on pupillary function

MDMA increased pupil size both at rest and after the light stimulus and lowered the constriction amplitude compared with placebo (Fig. 3, Table 1). The effect of MDMA on pupil size peaked (mean ± SEM) 2.3 ± 0.2 h after drug administration at the time of the maximal plasma concentration of MDMA and remained high over 6 h in parallel with plasma levels that also remained high over 6 h (Fig. 3A). The effect of MDMA on the constriction amplitude was maximal 1.7 ± 0.1 h after drug administration and decreased to baseline levels over 6 h (Fig. 3B) despite high plasma levels of MDMA. MDMA also prolonged the latency to the pupillary light reflex and shortened the recovery time of the pupillary light reflex response (Table 1).

Subjective effects of MDMA

MDMA produced significant subjective drug effects compared with placebo (Table 1). In parallel with the constriction amplitude the subjective peak effect was reached 1.5 ± 0.1 h after MDMA administration and completely reverted to baseline within 6 h, although the plasma levels of MDMA remained high (Fig. 3C). The effects of the pretreatments on the subjective response to MDMA are reported in detail elsewhere (Hysek et al. 2011, 2012a, b, d; Simmler et al. 2011). Briefly, reboxetine and duloxetine reduced the increases in "any subjective drug effect" and other aspects of the subjective effects of MDMA, whereas the other pretreatments overall had no effect on the subjective response to MDMA (Hysek et al. 2012c).

Effects of MDMA on blood pressure, heart rate, and body temperature

MDMA significantly increased blood pressure, heart rate, and body temperature compared with placebo (Table 1, Fig. 3D-F). Similar to the subjective effects, MDMA-induced increases in blood pressure and heart rate were short-lasting.

Pharmacokinetics of MDMA

Plasma MDMA concentrations are shown in Fig. 3. The peak plasma MDMA concentration was (mean ± SEM) 243 ± 6 ng/ml. The time to maximum plasma concentration was 2.5 ± 0.1 h.

Pharmacokinetic-pharmacodynamic and pharmacodynamic-pharmacodynamic associations

The relationships between the concentration of MDMA and its pharmacodynamic effects are shown in Fig. 4A-C. The average group pupil size was correlated with the average plasma levels of MDMA over time (R_s = 0.77, p < 0.01, n = 9), with moderate clockwise hysteresis (Fig. 4A). In contrast, the MDMA-induced reduction in constriction amplitude was not significantly associated with the plasma concentrations of MDMA (R_s = 0.43, p = 0.24, n = 9), attributable to pronounced clockwise hysteresis (Fig. 4B). There was

similar marked hysteresis and no association (R_s = 0.48, p = 0.17, n = 9) in the relationship between the concentration of MDMA and the group average of the subjective drug effects (Fig. 4C). The association between the average subjective effect and pupil size over time was relatively strong (R_s = 0.77, p < 0.01, n = 10), but hysteresis was observed in the relationship between subjective effects and pupil size over time (Fig. 4D), indicating that the subjective effects decreased more rapidly than the mydriasis associated with MDMA. In contrast, little or no hysteresis was observed in the plot of the relationship of subjective effects with constriction amplitude (Fig. 4E), indicating a closer association and more congruent subjective and dynamic pupillary effects of MDMA, also demonstrated by a very strong correlation between the means of these two effects over time (R_s = 0.96, p < 0.001, n = 10; Fig. 4E). Between-subjects correlations further showed that subjective effects were strongly correlated with reductions in the light reflex but not with pupil size (Table 2). There were similar strong associations between MDMA-induced reductions in constriction amplitude and changes in MAP, heart rate, and body temperature (R_s = 0.98, 0.92, 0.87; all p < 0.001, n = 10). MDMA-induced increases in blood pressure and heart rate did not correlate with the plasma concentrations of MDMA over time consistent with the reduced effect over time despite high plasma concentrations of MDMA (Fig. 3D and 3E).

The findings from the between-subjects analyses of the correlations between the plasma levels of MDMA and pharmacodynamic effects of MDMA for each time point (n = 80) are shown in Table 3. The MDMA-induced reductions in the constriction amplitude, pupil size after light, the increase in MAP, and the subjective effects were significantly and strongly associated with the plasma levels of MDMA (Table 3). Weaker correlations were also found between plasma levels of MDMA and pupil diameter, latency, and heart rate (Table 3). However, these associations were only observed until peak concentrations were reached. In contrast, recovery time and body temperature after MDMA administration were not or only weakly and inconsistently associated with plasma MDMA levels (Table 3).

The MDMA-induced reduction in pupil constriction amplitude was significantly greater in subjects with greater MDMA-induced increases in MAP (R_s = 0.56, p < 0.001, n = 80) or

more pronounced increases in heart rate (R_s = 0.30, p < 0.01, n = 80) as measured 1 h after MDMA administration. In contrast, MDMA-induced changes in the pupil size were not or only poorly associated with other autonomic changes across subjects.

Pupillary effects of reboxetine, duloxetine, clonidine, carvedilol, and doxazosin alone andon the pupillary response to MDMA

The peak effects of the pretreatments are shown in Table 4. The drug effects on pupil size over time for all five studies are shown in Fig. 5. Both reboxetine and duloxetine increased resting pupil size and pupil size after the light stimulus. Duloxetine also lowered the constriction amplitude (Table 4). The effect of the two monoamine uptake inhibitors on the static pupil diameter was similar in magnitude to the effect of MDMA (Table 4, Fig. 5A and B). In contrast, the effect of MDMA on the constriction amplitude was more pronounced. When duloxetine was administered together with MDMA, the drug effects on all static and dynamic parameters were non-additive and showed negative synergism, reflected by a significant pretreatment × MDMA interaction in the factorial ANOVA. Thus, duloxetine prevented the effect of MDMA on pupil function, reflected by the absence of a mydriatic effect of MDMA compared with baseline in the duloxetine-MDMA condition and compared with the duloxetine-placebo condition (Fig. 5B). Duloxetine also prevented the MDMA-induced impairment in the pupillary light reflex, although it had a similar effect when administered alone compared with placebo. The effects of reboxetine and MDMA on pupil size were also non-additive (Table 4, Fig. 5B). However, resting pupil size and pupil size after the light stimulus were significantly larger after reboxetine plus MDMA compared with MDMA alone. Reboxetine also failed to prevent the effect of MDMA on the pupillary light reflex. In the present study, reboxetine also reduced the cardio- and psychostimulant effects of MDMA (Hysek et al. 2011), and duloxetine nearly completely prevented the cardiovascular, psychotropic, and neuroendocrine effects of MDMA as reported elsewhere (Hysek et al. 2012b, 2012d). Clonidine reduced resting pupil size and size after the light stimulus (Table 4, Fig. 5C). This effect of clonidine was antagonistic and overall additive with

the effect of MDMA (Fig. 5C). Specifically, clonidine did not significantly reduce the effects of MDMA on any parameter of pupillary function, although it had significant effects alone and reduced the cardiovascular response to MDMA (Hysek et al. 2012a). Clonidine did not significantly reduce the mydriatic effects of MDMA, although it produced significant miosis. Clonidine also had no effects on the psychotropic response to MDMA as previously reported (Hysek et al. 2012a). Carvedilol did not alter the effects of MDMA on pupillary function. In contrast, carvedilol decreased the cardiostimulant and thermogenic effects of MDMA in the same subjects as reported elsewhere (Hysek et al. 2012c). Carvedilol alone decreased pupil size, reflected by a significant main effect of pretreatment in the ANOVA, but the reduction in pupil size after carvedilol-placebo treatment compared with the placebo-placebo condition (Fig. 5D) was not significant in the *post hoc* test. Doxazosin alone had no effect on pupil size compared with placebo, but nonsignificantly reduced the MDMA-induced increase in pupil size (Fig. 5E).

Discussion

In the present study, we showed that MDMA impaired the pupillary reflex response to light, including inducing a longer latency, reducing the constriction amplitude, and reducing the recovery time. MDMA produced mydriasis as previously documented using non-automated techniques (Farre et al. 2004, 2007; Kolbrich et al. 2008; Mas et al. 1999). MDMA also increased blood pressure, heart rate, and body temperature and produced positive mood effects as described in detail elsewhere (Hysek et al. 2012b, 2012d).

The analyses of the effects of MDMA over time showed a very strong correlation between the MDMA-induced reduction in constriction amplitude and the subjective and other autonomic effects of the drug. The MDMA-induced reduction in the pupillary light reflex normalized over 6 h, similar to the subjective and cardiostimulant drug effects that also largely disappeared over 6 h, although the plasma levels of MDMA remained high. Thus, the reduced reactivity of the pupil to light is relatively short-lasting and subject to acute pharmacological tolerance, similar to the subjective and cardiostimulant effects of MDMA.

Clinical examination of pupil function in cases of drug intoxication typically includes both an estimation of static pupil size and an assessment of the reactivity to a flashlight stimulus. With regard to MDMA intoxication, our findings suggest that the impaired reactivity to light indicates MDMA exposure within the past 1-4 h and is a marker for the acute subjective and autonomic effects of the drug. In contrast, mydriasis lasts at least 6-10 h (Farre et al. 2007; Mas et al. 1999), correlates best with the plasma MDMA concentration changes over time, and shows only moderate pharmacological tolerance. The mydriatic responses to two successive doses of MDMA separated by 24 h were similar, although the peak concentration after the second dose of MDMA increased by 29%, indicating some degree of tolerance (Farre et al. 2004). Although the mean group changes in pupil size over time reflected the concentration-time curve of MDMA, pupil size did not correlate well with the plasma concentrations of MDMA across subjects at various time points in our study or with MDMA plasma levels 1.25 h after drug administration in a previous study (Kolbrich et al. 2008). This is not surprising because the effects of MDMA on pupil size were maximal at single doses of 75 mg and did not further increase at 125 mg (Mas et al. 1999). Thus, the lack of an association is likely attributable to a ceiling effect of the plasma MDMA concentration-effect curve. In contrast, dynamic impairments of the pupil light reflex response were significantly associated with plasma MDMA levels or the cardiostimulant effects of MDMA across subjects. Evaluating the dynamic pupillary response to light may therefore be a better estimation of the time and amount of exposure to MDMA than static pupil size.

Both sympathetic and parasympathetic innervations contribute to the regulation of pupil size and the reflex response (Loewenfeld 1999). At the level of the iris, the latency to and amplitude of the reflex response are mainly determined by parasympathetic activity (Heller et al. 1990), whereas redilation is controlled by sympathetic inputs (Loewenfeld 1999; Morley et al. 1991). Additionally, parasympathetic function is under tonic noradrenergic inhibition centrally at the level of the Edinger-Westphal nucleus where the sympathetic stimulation of α_2-adrenergic receptors may lower parasympathetic output, resulting in “pseudoanticholinergic” mydriasis (Phillips et al. 2000a; Siepmann et al. 2007; Szabadi and

Bradshaw 1996). Furthermore, the serotonin system is implicated in pupillary function, possibly via 5-HT_{1A}-mediated stimulation of the release of norepinephrine and consequent activation of α_2-adrenergic receptors (Prow et al. 1996). MDMA mainly releases serotonin and norepinephrine (Liechti and Vollenweider 2001; Rothman et al. 2001; Verrico et al. 2007). Because MDMA affected both the parasympathetic and sympathetic aspects of the pupillary reflex response all of the aforementioned mechanisms may be involved in the effects of MDMA on pupillary function.

The norepinephrine transporter inhibitor reboxetine significantly increased pupil diameter at rest and after light consistent with previous studies (Szabadi et al. 1998; Theofilopoulos et al. 1995). Reboxetine did not reduce the mydriatic response to MDMA, but the effects of the two drugs on pupil size were subadditive. The interactive effect of reboxetine and MDMA on pupil size indicates that MDMA produces part of its effects on pupil size through the transporter-mediated release of norepinephrine, which is inhibited by reboxetine (Hysek et al. 2011). This finding is consistent with the attenuation of the cardio- and psychostimulant effects of MDMA by reboxetine (Hysek et al. 2011) and supports the view that norepinephrine is involved in the stimulant effects of MDMA.

The α_1-adrenergic receptor inhibitor doxazosin did not affect pupillary function when administered alone but nonsignificantly reduced the mydriatic response to MDMA. Prazosin did not antagonize mydriasis induced by norepinephrine or phenylephrine in anesthetized cats (Hey et al. 1988; Koss et al. 1988). The data indicate that α_1-adrenergic receptors in the iris may only minimally contribute to mydriasis induced by systemically administered sympathomimetic drugs and that central parasympathetic inhibition may be more relevant.

The α_1-β-adrenergic receptor inhibitor carvedilol had no significant effect on pupil size compared to placebo, consistent with earlier work (Hirohashi et al. 1990) and the absence of effects of the β-adrenergic receptor blocker propranolol on pupillary function (Koudas et al. 2009). Carvedilol did not affect the mydriatic response to MDMA, but it reduced other autonomic effects of MDMA, including increases in blood pressure and body temperature (Hysek et al. 2012c).

Clonidine decreased pupil diameter and enhanced the pupillary reflex, consistent with its known sympatholytic effects (Clifford et al. 1982; Morley et al. 1991; Phillips et al. 2000b, c). Clonidine also lowered the plasma concentrations of norepinephrine and blood pressure in the subjects of the present study (Hysek et al. 2012a). The effect of clonidine on pupil function is thought to involve the stimulation of α_2-adrenergic receptors on central noradrenergic neurons, leading to decreased sympathetic outflow to the iris. The enhancement of the parasympathetic light reflex is consistent with clonidine-induced disinhibition of the noradrenergic central control of parasympathetic outflow (Phillips et al. 2000b). Despite its significant sympathicolytic effects (Hysek et al. 2012a), clonidine failed to significantly reduce the effects of MDMA on pupillary function. Moreover, clonidine did not reduce the MDMA-induced increase in norepinephrine or blood pressure to the same extent as it reduced these parameters when administered alone (Hysek et al. 2012a). Thus, the sympatholytic effects of clonidine and sympathomimetic effects of MDMA were antagonistic in an additive manner, without evidence of interactive effects of the two drugs. The findings indicate that α_2-adrenergic receptors and the vesicular release of norepinephrine are not critically involved in the pharmacological effects of MDMA.

The serotonin and norepinephrine transporter inhibitor duloxetine increased resting pupil diameter, prolonged the latency to the light reflex, and reduced the reaction to light. Identical effects on pupillary function have been reported for the serotonin and norepinephrine transporter inhibitor venlafaxine (Bitsios et al. 1999; Siepmann et al. 2007). The effects of duloxetine on pupillary function likely involve both enhanced noradrenergic neurotransmission in the iris and central sympathomimetic inhibition of the parasympathetic outflow to the iris. Additionally, the involvement of the serotonin system is likely. Serotonin releasers, including fenfluramine (Kramer et al. 1973), meta-chlorophenylpiperazine (Benjamin et al. 1997), and MDMA, and serotonin transporter inhibitors (Nielsen et al. 2010; Noehr-Jensen et al. 2009; Schmitt et al. 2002) cause mydriasis. Citalopram and paroxetine have also been shown to reduce the constriction amplitude (Nielsen et al. 2010; Noehr-Jensen et al. 2009), similar to previous observations with duloxetine. Duloxetine may

therefore exert its effects on pupillary function via both noradrenergic and serotonergic mechanisms. Although both duloxetine and MDMA produced mydriasis, pupil size did not further increase after administration of both drugs, suggesting interactive effects of the two drugs. Moreover, duloxetine almost completely prevented the effects of MDMA on the light reflex. Duloxetine also markedly inhibited the cardiostimulant, psychotropic, and neuroendocrine responses to MDMA in the same subjects (Hysek et al. 2012b, 2012d). Selective serotonin transporter inhibitors including citalopram, fluoxetine, and paroxetine, have previously been shown to attenuate the physiological and psychological effects of MDMA in humans (Farre et al. 2007; Liechti et al. 2000; Liechti and Vollenweider 2000; Tancer and Johanson 2007). Notably, paroxetine prevented the mydriatic effects of MDMA (Farre et al. 2007). Together with the interactive effects of duloxetine and MDMA in the present work, the findings provide strong support for a role of serotonin in the mechanism of action of MDMA. The reduction of the effects of MDMA on the pupil light reflex by duloxetine but not reboxetine supports a central modulatory role of serotonin in the effects of MDMA on pupillary function, possibly involving central serotonergic potentiation of noradrenergic outflow (Prow et al. 1996).

In the present study, we also described the basic characteristics of the pupillary reflex. We assessed pupillary function under standardized dark-light conditions, similar to other studies of the autonomic effects of pharmaceuticals (Bitsios et al. 1999; Nielsen et al. 2010; Noehr-Jensen et al. 2009; Phillips et al. 2000c). The values of the latency to the light reflex and constriction amplitude obtained in the present study were similar to those measured under daylight conditions with the same pupillometer (Taylor et al. 2003), indicating that these parameters may not be critically affected by the light conditions. Overall, our data indicate that the constriction of the pupil represents a measure that is sensitive to pharmacological interventions and may be relatively insensitive to changes in light conditions compared with measures of pupil size.

In summary, MDMA increased pupil size and reduced the response to light. The MDMA-induced prolongation of the latency to the light reflex and reduction in light-induced miosis

indicate indirect central parasympathetic inhibition. The faster recovery reflects increased direct sympathomimetic action. Both reboxetine and duloxetine interacted with the effects of MDMA on static and dynamic measures of pupillary function supporting a role for both norepinephrine and serotonin in the effects of MDMA on pupillary function. MDMA-induced mydriasis was associated with the plasma concentration-time curve of MDMA. The reduced miotic response to light was highly correlated with the subjective and cardiostimulant effects of MDMA, and demonstrated acute pharmacological tolerance.

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Fehler! Es ist nicht möglich, durch die Bearbeitung von Feldfunktionen Objekte zu erstellen.

Figure 1. Study diagram.

Fehler! Es ist nicht möglich, durch die Bearbeitung von Feldfunktionen Objekte zu erstellen.

Figure 2. Schematic drawing of the light reflex response. MAX represents dark-adapted resting pupil size before the light stimulus. Latency represents the time of the onset of constriction. MIN represents minimal pupil size after the light stimulus. The constriction amplitude was calculated as MAX-MIN. The 75% recovery time is the time to recover 75% of the initial resting pupil size after reaching MIN.

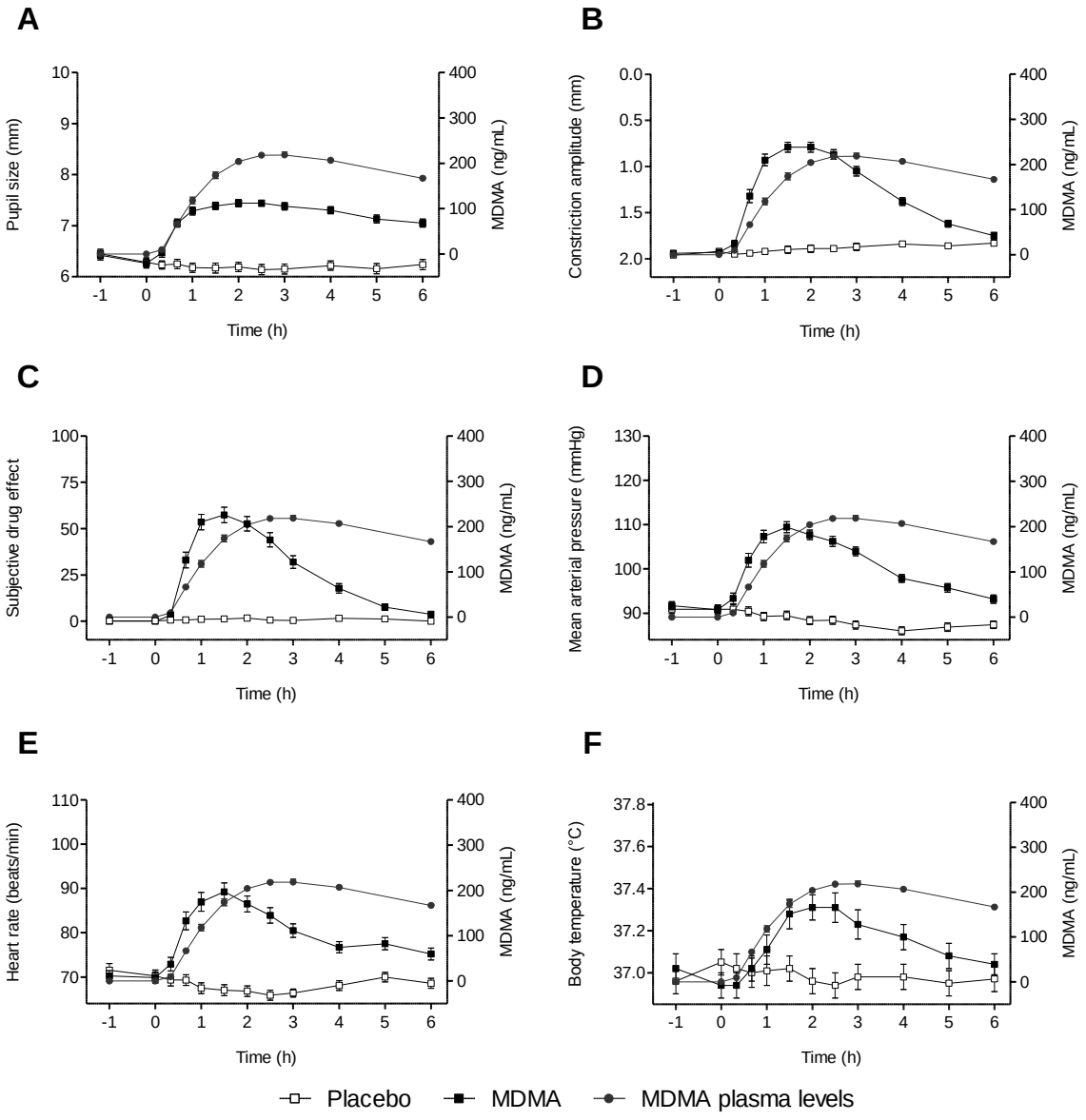

Figure 3. Acute effects of MDMA on pupil function. Values are expressed as the mean ± SEM of 80 subjects. MDMA increased resting pupil size compared with placebo (**A**). The mydriatic effect of MDMA remained high in parallel with the plasma concentration of MDMA. MDMA reduced the pupil constriction amplitude compared with placebo and this effect decreased more rapidly than the plasma concentration of MDMA (**B**). The subjective (**C**), cardiovascular (**D-E**), and thermogenic (**F**) effects of MDMA also disappeared within 6 h when the plasma concentrations of MDMA were still high.

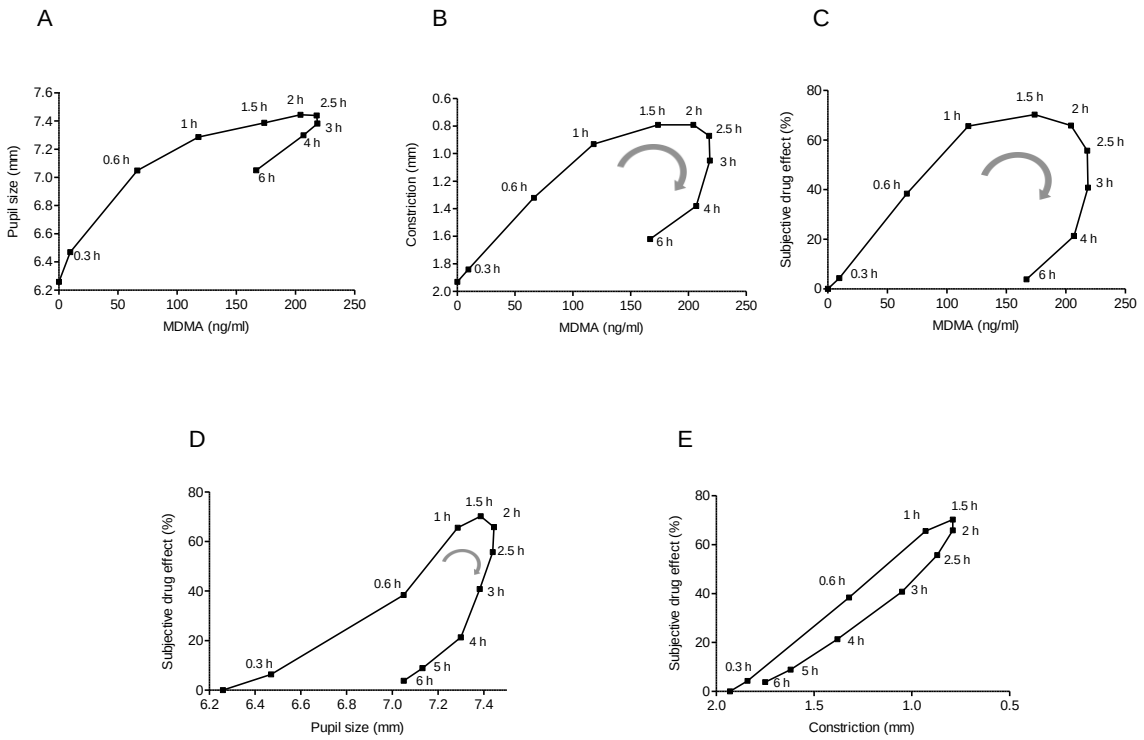

Figure 4. Pharmacokinetic-pharmacodynamic relationship. MDMA effects plotted against the plasma concentrations of MDMA (**A-C**). The values are expressed as the means of 80 subjects, with SEM omitted for clarity. The times of pupillometry and blood sampling are noted next to each point in minutes or hours after MDMA administration. While pupil size remained high (**A**), constriction amplitude (**B**) and the subjective effect (**C**) returned to baseline within 6 h when MDMA concentrations remained high. This clockwise hysteresis was moderate for the mydriatic effect of MDMA, reflecting well the plasma concentration of MDMA (**A**), but pronounced for the impairment in the pupillary reflex response (**B**) and subjective effect of MDMA (**C**). The subjective effect of MDMA returned to baseline faster than the mydriatic response to MDMA (**D**). In contrast, the time course of the subjective effect of MDMA was more congruent with the time course of the MDMA-induced impairment in constriction amplitude (**E**).

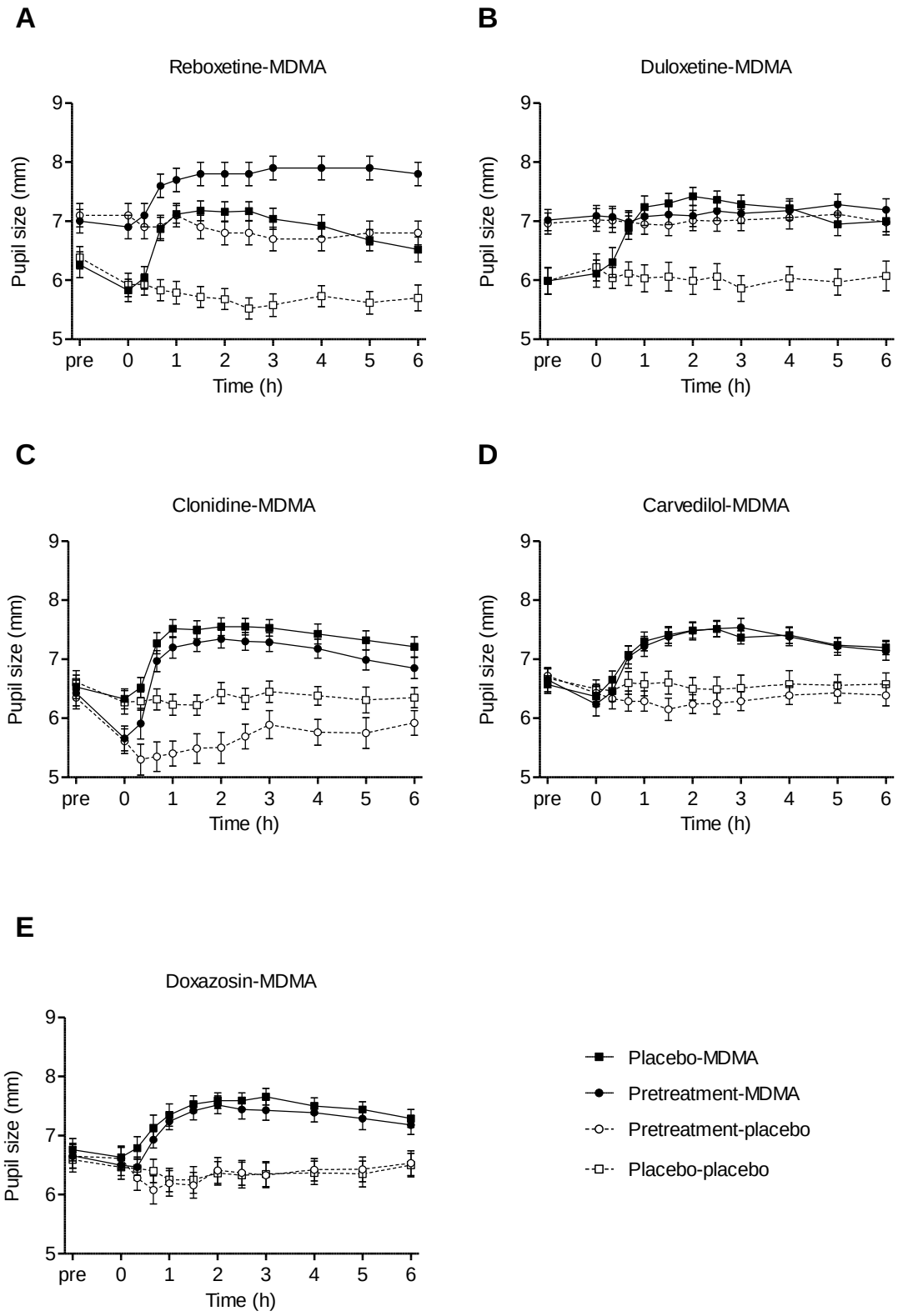

Figure 5. Effects of pretreatments, MDMA, and the combination of the pretreatments with MDMA and placebo on pupil size over time. MDMA increased pupil size compared with placebo in all of the studies (**A-E**). The pretreatment with reboxetine increased pupil size to a similar extent as MDMA alone (**A**). The effect of MDMA on pupil diameter after reboxetine

pretreatment compared with reboxetine was significantly smaller than the effect of MDMA compared with placebo (**A**). Duloxetine increased pupil size similar to reboxetine and MDMA (**B**). Duloxetine pretreatment prevented the further increase in pupil size induced by MDMA administration (**B**). Clonidine significantly reduced pupil diameter (**C**). The effects of clonidine and MDMA on pupil size were additive (**C**) (Table 2). Carvedilol decreased pupil size (**D**). Similar to the effects of clonidine and MDMA, the effects of carvedilol and MDMA on pupil size were additive (**D**). Doxazosin alone had no effect on pupil size compared with placebo, but it tended to nonsignificantly attenuate the mydriatic effect of MDMA (**E**). The data are expressed as mean ± SEM values in 16 subjects per study.

Table 1. Effects of MDMA

		Placebo (mean±SEM)	MDMA (mean±SEM)	$F_{1,79}$=	P<
Pupil size (mm)	E_{max}	6.60±0.09	7.58±0.07	288.1	0.001
Pupil size after light (mm)	E_{max}	4.76±0.09	6.86±0.09	646.9	0.001
Constriction amplitude (mm)	E_{min}	1.76±0.06	0.81±0.12	328.0	0.001
Latency (sec)	E_{max}	0.25±0.00	0.33±0.02	16.2	0.001
Recovery time (sec)	E_{min}	1.74±0.06	1.17±0.07	57.3	0.001
Subjective drug effect (%maximum)	E_{max}	3.5±1.7	81.0±2.8	901.2	0.001
Mean arterial pressure (mmHg)	E_{max}	95.0±1.0	114.5±1.2	339.7	0.001
Heart rate (beats/min)	E_{max}	76.0±1.2	96.2±1.9	138.1	0.001
Body temperature (°C)	E_{max}	37.3±0.1	37.6±0.1	26.2	0.001

N=80 (values from all five studies were pooled)

Table 2. Correlations between MDMA-induced changes in pupillary function and the subjective drug effect

	t=0	t=20 min	t=40 min	t=1h	t=1.5h	t=2h	t=2.5h	t=3h	t=4h	t=6h
Pupil size (mm)	NS	NS	0.31	0.27	NS	NS	NS	NS	NS	NS
Pupil size after light (mm)	NS	NS	**0.62**	**0.51**	**0.42**	0.26	0.27	NS	NS	NS
Constriction amplitude (mm)	NS	NS	**-0.74**	**-0.61**	**-0.41**	-0.28	-0.28	-0.23	-0.23	NS
Latency (sec)	NS	NS	**0.46**	0.29	NS	NS	NS	NS	NS	NS
Recovery time (sec)	NS	NS	**-0.42**	-0.31	-0.32	-0.22	**-0.38**	-0.23	-0.28	-0.32

Values are Spearmann correlation coefficients for significant correlations (P<0.05; P<0.001in bold). NS, not significant. N=80.

Table 3. Correlations between the effects of MDMA and plasma concentrations of MDMA

	t=0	t=20 min	t=40 min	t=1h	t=1.5h	t=2h	t=2.5h	t=3h	t=4h	t=6h
Pupil size (mm)	NS	**0.46**	**0.45**	0.35	0.27	NS	NS	NS	NS	NS
Pupil size after light (mm)	NS	**0.50**	**0.64**	**0.57**	**0.54**	**0.53**	**0.49**	**0.47**	0.34	NS
Constriction amplitude (mm)	NS	-0.28	**-0.40**	**-0.55**	**-0.53**	**-0.60**	**-0.55**	**-0.65**	**-0.48**	-0.28
Latency (sec)	NS	0.23	**0.46**	0.33	0.35	NS	0.25	0.25	NS	0.25
Recovery time (sec)	NS	NS	-0.32	NS	-0.26	NS	**-0.37**	-0.26	-0.26	NS
Subjective drug effect	NS	NS	**0.68**	**0.56**	**0.37**	0.31	**0.44**	0.34	**0.46**	0.31
Mean arterial pressure (mm Hg)	NS	0.22	**0.69**	**0.60**	**0.47**	0.35	0.33	0.29	**0.39**	NS
Heart rate (beats/min)	NS	NS	**0.61**	**0.47**	**0.46**	0.32	NS	NS	NS	NS
Body temperature (°C)	NS	-0.24	NS	NS	NS	NS	NS	NS	NS	NS

Values are Spearmann correlation coefficients for significant correlations (P<0.05; P<0.001in bold) between MDMA-induced pharmacodynamic changes and plasma levels of MDMA. NS, not significant. N=80

Table 4. Effects of pretreatments, of MDMA, and of the combination on pupil function

		mean±SEM values				main effect of MDMA		main effect of pretreatment		Pretreatment × MDMA	
		Placebo	Pretreatment	MDMA	Pretreatment-MDMA	$F_{1,15}$ =	P<	$F_{1,15}$ =	P<	$F_{1,15}$ =	P<
Pupil size (mm)											
Reboxetine	E_{max}	6.15±0.19	7.22±0.20***	7.32±0.17***	8.08±0.18*** ###	124.8	0.001	160.4	0.001	10.7	0.01
Duloxetine	E_{max}	6.55±0.21	7.30±0.19***	7.52±0.16***	7.65±0.16***	58.9	0.001	37.0	0.001	11.2	0.01
Clonidine	E_{min}	5.86±0.20	4.93±0.22***###	7.06±0.17***	6.72±0.15***	87.9	0.001	59.3	0.001	7.8	0.05
	E_{max}	6.75±0.16	6.35±0.22*** ###	7.65±0.15***	7.46±0.16***	68.7	0.001	12.7	0.01	2.8	NS
Carvedilol	E_{max}	6.88±0.19	6.66±0.16###	7.63±0.13***	7.66±0.14***	99.5	0.001	8.0	0.05	1.2	NS
Doxazosin	E_{max}	6.67±0.20	6.69±0.18###	7.78±0.12***	7.53±0.14***	58.2	0.001	2.7	NS	2.2	NS
Pupil size after light (mm)											
Reboxetine	E_{max}	4.23±0.17	5.50±0.22*** ###	6.65±0.23***	7.37±0.21*** ###	289.1	0.001	129.9	0.001	9.3	0.01
Duloxetine	E_{max}	4.78±0.22	5.96±0.26*** ###	6.94±0.19***	6.38±0.22*** ##	108.0	0.001	10.9	0.01	84.2	0.001
Clonidine	E_{min}	3.84±0.64	3.15±0.63** ###	5.31±0.83***	5.08±0.97***	93.1	0.001	24.0	0.001	5.2	0.05
	E_{max}	4.80±0.16	4.47±0.22###	7.01±0.23***	6.83±0.24***	211.2	0.001	8.9	0.01	1.2	NS
Carvedilol	E_{max}	5.10±0.25	4.77±0.17###	6.82±0.17***	7.04±0.16***	202.3	0.001	0.4	NS	2.3	NS
Doxazosin	E_{max}	4.88±0.19	4.88±0.18###	6.86±0.15***	6.79±0.16***	169.4	0.001	0.1	NS	0.2	NS
Constriction amplitude (sec)											
Reboxetine	E_{min}	1.74±0.06	1.62±0.07###	0.60±0.12***	0.67±0.10***	71.8	0.001	0.7	NS	3.0	NS
Duloxetine	E_{min}	1.62±0.05	1.27±0.09** ###	0.52±0.11***	1.18±0.06*** ###	63.9	0.001	12.4	0.01	71.2	0.001
Clonidine	E_{min}	1.81±0.05	1.63±0.08###	0.58±0.11***	0.50±0.09***	72.0	0.001	7.6	0.05	1.0	NS
Carvedilol	E_{min}	1.65±0.10	1.75±0.06###	0.68±0.11***	0.49±0.08***	99.5	0.001	0.7	NS	4.2	NS
Doxazosin	E_{min}	1.76±0.06	1.69±0.05###	0.81±0.12***	0.78±0.09***	79.6	0.001	1.2	NS	0.3	NS
Latency (sec)											
Reboxetine	E_{max}	0.244±0.006	0.254±0.007 ##	0.303±0.011***	0.306±0.015***	14.8	0.001	2.8	NS	0.0	NS
Duloxetine	E_{max}	0.245±0.005	0.266±0.007	0.423±0.088*	0.275±0.005	4.8	0.05	2.2	NS	3.8	0.07
Clonidine	E_{max}	0.252±0.011	0.251±0.023##	0.297±0.033**	0.308±0.061***	30.9	0.001	0.7	NS	0.4	NS
Carvedilol	E_{max}	0.260±0.007	0.262±0.009##	0.304±0.016**	0.303±0.010##	15.5	0.001	0.0	NS	0.0	NS
Doxazosin	E_{max}	0.253±0.008	0.249±0.006#	0.325±0.037*	0.291±0.009	10.4	0.01	1.1	NS	1.1	NS

*<0.05, **<0.01, ***<0.001, compared with Placebo; #<0.05, ##<0.01, ###<0.001, compared with MDMA; NS, non significant