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Protracted manifestations of acute dependence after a single morphine exposure

Patrick E. Rothwell,

Department of Neuroscience, University of Minnesota, 321 Church St SE, Minneapolis, MN 55455, USA

Mark J. Thomas, and

Department of Neuroscience, University of Minnesota, 321 Church St SE, Minneapolis, MN 55455, USA; Department of Psychology, University of Minnesota, 75 East River Rd, Minneapolis, MN 55455, USA

Jonathan C. Gewirtz

Department of Neuroscience, University of Minnesota, 321 Church St SE, Minneapolis, MN 55455, USA; Department of Psychology, University of Minnesota, 75 East River Rd, Minneapolis, MN 55455, USA

Abstract

Rationale—Acute opiate exposure produces a state of dependence in humans and animals, which is revealed by signs and symptoms of withdrawal precipitated by opioid receptor antagonists. The physiological changes that underlie this state of acute dependence develop rapidly and can persist long after the end of chronic opiate exposure.

Objectives—The purpose of this investigation was to determine the persistence of acute dependence after a single morphine exposure in rodents, focusing on changes in behavior thought to reflect the negative emotional consequences of withdrawal.

Methods—The acoustic startle reflex and conditioned place aversion were measured following naloxone administration at different time points after a single morphine exposure.

Results—Naloxone administration produced significant potentiation of acoustic startle—a form of anxiety-like behavior—for at least 80 days after one exposure to morphine. In contrast, naloxone produced a conditioned place aversion 24 h but not 20 days after one morphine exposure.

Conclusions—Together with existing literature, these results suggest acute as well as chronic opiate exposure leave rodents persistently vulnerable to express anxiety-like behavior in response to opioid receptor antagonists or stressful experience. The adaptations in brain function that underlie this protracted state of dependence may provide a foundation for the escalation of withdrawal severity that develops over repeated opiate exposure, and increase the likelihood of progression from casual drug use to compulsive drug abuse.

Keywords

Addiction; Abstinence; Morphine; Naloxone; Withdrawal; Anxiety; Startle; Aversion

Introduction

The development of addiction involves a cascade of adaptations in brain function and behavior (Hyman et al. 2006; Kalivas and O'Brien 2008; Koob and Le Moal 2008; Robinson and Berridge 2003), which begin with the very first exposure to an abused drug (Kim et al. 2004; Saal et al. 2003; Ungless et al. 2001; Vanderschuren et al. 2001; Vanderschuren et al. 1999). The adaptive changes caused by acute exposure to morphine and other opiates can be revealed by the administration of opioid receptor antagonists, which precipitate the signs and symptoms of withdrawal in both humans and animals after as little as one opiate exposure (for review, see Harris and Gewirtz 2005). Manifestations of this state of "acute dependence" include physical signs and negative emotional symptoms in humans, as well as a variety of neural, behavioral, and endocrine changes in animals. This body of evidence indicates just a single experience with an opiate is sufficient to initiate adaptive changes in physiology that may contribute to the development and progression of addiction.

Among the various facets of the opiate withdrawal syndrome, negative emotional states (like anxiety, dysphoria, and anhedonia) are thought to play a primary role in motivating compulsive drug use (Baker et al. 2004; Koob and Le Moal 2008; Smith and Aston-Jones 2008). Studies of acute dependence in rodents have documented changes in behavior that may correspond to the negative emotional states associated with opiate withdrawal in humans. This includes anxiety-like behavior in a number of paradigms, including the elevated plus maze (Zhang and Schulteis 2008), ultrasonic vocalizations (Kalinichev and Holtzman 2003), and acoustic startle (Harris and Gewirtz 2004). Other behavioral measures like conditioned place aversion (Azar et al. 2003), suppression of operant responding (Adams and Holtzman 1990), and elevated thresholds for self-stimulation (Liu and Schulteis 2004) may be related to anxiety or other emotional components of withdrawal, such as anhedonia or dysphoria. These "affective signs" of withdrawal are elicited by lower doses of antagonist than classic physical manifestations of withdrawal (Gellert and Sparber 1977; Higgins and Sellers 1994; Schulteis et al. 1994), and are also mediated by distinct neural substrates (Koob et al. 1992), including portions of the "extended amygdala" (Koob and Le Moal 2008).

While it is clear that acute dependence develops rapidly after as little as one opiate exposure (e.g., Eisenberg 1982), relatively less is known about how long this state of dependence persists. Human studies have documented aspects of acute dependence that last at least 24 h, and even up to 1 week after a single opiate exposure (Nutt and Jasinski 1974). In rodents, signs of withdrawal can still be precipitated when naloxone is administered 24 hours after a single morphine exposure (Araki et al. 2004; Eisenberg 1982; Parker and Joshi 1998; Rothwell et al. 2009), but these effects have not been examined beyond 48 h (Gellert and Sparber 1977; Parker et al. 2002). In contrast, chronic administration of opiates to rodents and primates has been shown to increase sensitivity to opioid receptor antagonists for weeks after terminating drug exposure (Celerier et al. 2001; Goldberg and Schuster 1969). It is not known whether these persistent aspects of dependence require extensive opiate exposure or if more limited drug exposure regimens also cause long-lasting changes in physiology.

Here, we explored the persistence of acute dependence in rats by administering naloxone at different time points after a single morphine exposure and measuring two affective signs of withdrawal. The first, potentiation of the acoustic startle reflex, occurs in situations that provoke fear and anxiety in humans, primates, and rodents (Davis et al. 2010). Startle potentiation is also observed during spontaneous and precipitated withdrawal from acute opiate exposure (Cabral et al. 2009; Harris and Gewirtz 2004; Kalinichev and Holtzman 2003; Rothwell et al. 2009). This effect is attenuated by anxiolytic drugs (Harris and

Gewirtz 2004; Rothwell et al. 2009) and involves portions of the extended amygdala (Cabral et al. 2009; Harris et al. 2006), suggesting it represents a form of anxiety-like behavior. The second measure, conditioned place aversion (CPA), has been extensively utilized to study drug withdrawal as well as other aversive states in rodents (Tzschentke 1998; 2007). Previous research has shown naloxone administration causes both startle potentiation and CPA for at least 24 h after a single morphine exposure (Parker and Joshi 1998; Rothwell et al. 2009), a pattern we confirm in the present report. We also find that CPA is no longer observed when naloxone is administered 20 days after one morphine injection. In contrast, startle potentiation after naloxone administration persists for at least 80 days after a single morphine exposure—a remarkably enduring imprint of extremely limited drug exposure.

Materials and methods

Subjects

Male Sprague–Dawley rats (Harlan), weighing 175–199 g upon arrival, were housed in groups of four to five in metal cages with a 12-h light/dark cycle (light on 0800–2000 hours) and free access to food and water except during testing. The rats were allowed to acclimate to housing conditions for 2 weeks after arrival and were gently handled for two consecutive days prior to any testing or drug treatment. All procedures conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Minnesota Institutional Animal Care and Use Committee.

Drugs

Morphine sulfate was provided by the National Institute on Drug Abuse (Rockville, MD). Naloxone hydrochloride was obtained from Sigma (St. Louis, MO). All drugs were dissolved in 0.9% saline and injected s.c. in a volume of 1 mL/kg body weight. Drug doses are expressed as the weight of the salt and were selected on the basis of our previous studies (Harris et al. 2006; Harris and Gewirtz 2004; Harris et al. 2004; Harris et al. 2008; Rothwell et al. 2010; Rothwell et al. 2009).

Acoustic startle

The apparatus for testing acoustic startle has been described previously (Engelmann et al. 2009; Rothwell et al. 2009). To acclimatize animals to the experimental procedure, acoustic startle was tested on each of 2 days prior to testing with naloxone. For each session, rats were placed in the startle chambers for a 5-min habituation period, and then presented with 40 startle stimuli (20 each at 95 or 105 dB in semi-random order) with a 30-s interstimulus interval. For startle tests 24 h after morphine exposure (Fig. 1), a single morphine (10 mg/kg) or saline injection was given after the second startle acclimation session, with the naloxone test conducted 24 h later. For time points greater than 24 h after morphine exposure (Fig. 2), all animals received a single morphine injection (10 mg/kg) after the second day of handling, and startle acclimation did not begin until 2 days before the naloxone test. On the day of naloxone test, baseline startle was first measured prior to injection. Later in the same day, naloxone (0.8–8 mg/kg) was administered in the animal colony and rats were immediately transported to startle chambers for a test session.

Place conditioning

As previously described (Rothwell et al. 2009), the place conditioning apparatus consisted of a rectangular plastic cage divided in two by a central partition, with a distinct floor texture and wall color on each side (metal bars and white walls versus wire mesh and striped walls). Each experiment began with a 10-min baseline session in which the rats were free to move between both sides of the apparatus. The animals used in this study spent an average of 326

s (54.3%) on the bar side during the baseline session. The side of the apparatus paired with naloxone administration was counterbalanced (Carlezon 2003), yielding an unbiased procedure in which rats spent ~50% of the baseline session on the side to be paired with naloxone.

Conditioning sessions began 24 h after the baseline session. Each animal was conditioned once with saline and once with naloxone (2.5 mg/kg) on separate days in counterbalanced order and confined to the appropriate side of the apparatus for 30 min after each injection. A single morphine injection (10 mg/kg) was given either 24 h or 20 days prior to the naloxone-conditioning session. A 10-min test session was conducted 24 h after the last conditioning session, in which rats were again free to move between both sides of the apparatus.

Data analysis

As startle results were similar at both stimulus intensities (95/105 dB—see text as well as Harris and Gewirtz 2004), startle data were collapsed across intensity for statistical analysis unless otherwise noted. Changes in startle following naloxone administration were calculated as percent change from baseline on the same day (Walker and Davis 2002). Place conditioning data are expressed in terms of percent time spent on the drug-paired side, rather than using a difference score measured in seconds, because percentage measures are relatively independent of the length of the conditioning session and thus facilitate comparison across studies (Rothwell et al. 2009). Data were analyzed by analysis of variance (ANOVA) using SPSS (version 13.0) with a type I error rate of $\alpha=0.05$ (two-tailed) and are presented in figures as mean $\pm$ SEM. Group sizes are indicated in the text and figure legends.

Results

To further characterize the conditions that produce startle potentiation 24 h after a single morphine exposure (Rothwell et al. 2009), we injected separate groups of animals with several different doses of naloxone (0.8, 2.5, or 8 mg/kg) or saline at this time point. Baseline startle amplitudes (saline, 29.6 ± 3.1 ; 0.8 mg/kg, 35.6 ± 7.8 ; 2.5 mg/kg, 25.9 ± 3.3 ; 8 mg/kg, 25.3 ± 2.8) did not differ significantly between groups prior to naloxone injection ($F_{3,20}=1.02$, $p=0.41$). Following naloxone injection (Fig. 1), there was a significant linear effect of naloxone dose ($F_{1,20}=8.10$, $p=0.001$), and planned comparisons indicated that both 2.5 and 8 mg/kg naloxone caused significant startle potentiation ($p<0.05$). As 2.5 mg/kg was the minimum dose of naloxone that produced significant startle potentiation, we utilized this dose in all subsequent experiments.

Previous studies from our lab and others indicate that injection with 2.5 mg/kg naloxone does not alter startle amplitude in rodents that have never been exposed to morphine (Davis et al. 1988; Glover and Davis 2008; Harris et al. 2004; Hijzen et al. 1995; Rothwell et al. 2009). To confirm this in the present study, two control groups were given a single injection of saline rather than morphine. The following day, one morphine-naïve group was injected with 2.5 mg/kg naloxone, while the second morphine-naïve group received a second saline injection. Baseline startle amplitudes (saline, 47.4 ± 5.1 , $n=8$; naloxone, 47.8 ± 3.2 , $n=8$) did not differ between groups ($F_{1,14}<1$). Startle values following injection were similar to baseline (saline, $-7.4\pm3.9\%$; naloxone, $1.0\pm4.9\%$) and not significantly different between groups ($F_{1,14}=1.84$, $p=0.20$). The fact that 2.5 mg/kg naloxone does not affect startle in morphine-naïve animals, but significantly potentiates startle 24 h after a single morphine exposure, demonstrates the defining feature of acute opiate dependence: an enhanced sensitivity to the behavioral effects of an opioid receptor antagonist. As neither saline nor naloxone injection altered startle magnitude at this time point in morphine-naïve animals, we

did not include these negative control groups in subsequent experiments examining the time course of acute dependence.

While it is well established that acute dependence can be induced rapidly following a single opiate exposure (e.g., Eisenberg 1982), it is not clear whether this state of acute dependence persists for more than 48 h (Gellert and Sparber 1977; Parker et al. 2002). To address this issue, we next administered 2.5 mg/kg naloxone at different time points after a single injection of 10 mg/kg morphine (Fig. 2). For comparison, data from Fig. 1 using this dose of naloxone are reproduced as the 1-day time point in Fig. 2, showing significant startle potentiation after a single morphine exposure ($F_{1,10}=6.61$, $p=0.028$). This effect persisted for 5 days ($F_{1,10}=12.89$, $p=0.005$), 10 days ($F_{1,23}=5.17$, $p=0.033$), 20 days ($F_{1,10}=24.25$, $p=0.001$), and up to 80 days ($F_{1,21}=6.58$, $p=0.018$)—the longest time point tested. There were no significant differences in baseline startle amplitude at any time point between groups receiving naloxone and their corresponding saline control group (Table 1).

Although there was a numerical tendency for the magnitude of startle potentiation to increase from 1 to 20 days after morphine before decreasing somewhat at 80 days, neither the naloxone $\times$ day interaction ($F_{4,74}<1$) nor the quadratic trend across days in the naloxone groups ($p=0.25$) reached statistical significance. This pattern could be explained if startle is maximally potentiated by this dose of naloxone, leading to a ceiling effect that precludes detection of time-related changes in naloxone sensitivity. To assess this possibility, we separately analyzed startle data for the two different stimulus intensities (95 and 105 dB) from the naloxone group on day 20. Baseline startle amplitudes in response to 95 and 105 dB stimuli were 36.2 and 42.9 units, respectively. Startle amplitudes increased to 55.0 and 65.9 units following the administration of naloxone, which represent increases of 51.1% and 54.6% relative to baseline. The fact that raw startle amplitude is still larger at 105 than 95 dB following the administration of naloxone indicates startle is not maximally potentiated and can still be further enhanced under these conditions. Furthermore, a lower dose of naloxone (0.8 mg/kg) that does affect startle 1 day after morphine (Fig. 1) still had no significant effect on startle 20 days after morphine (naloxone, $10.6\pm14.5\%$, $n=6$; saline, $18.5\pm14.7\%$, $n=6$; $F_{1,10}<1$), arguing against any time-related changes in naloxone sensitivity.

Naloxone also produces a CPA when administered 24 h after a single morphine exposure (Araki et al. 2004; Jin et al. 2004; Parker and Joshi 1998; Rothwell et al. 2009), but the persistence of this effect has only been tested up to 48 h post-morphine (Parker et al. 2002). We thus examined whether one place conditioning session with 2.5 mg/kg naloxone would cause CPA 20 days after a single exposure to 10 mg/kg morphine, compared to the CPA produced 24 h after morphine exposure (Fig. 3a). As expected, this dose of naloxone caused robust CPA 1 day after morphine ($F_{1,15}=35.6$, $p<0.001$), consistent with previous studies cited above. However, this effect was no longer observed 20 days after a single morphine exposure ($F_{1,23}<1$). An additional control group ($n=16$) was conditioned with naloxone 24 h after a single saline injection. No significant effects were observed (baseline, $50.0\pm3.1\%$; test, $46.8\pm4.8\%$; $F_{1,15}<1$), again demonstrating that a single morphine exposure enhances sensitivity to this dose of naloxone—the defining feature of acute dependence.

To verify the efficacy of naloxone 20 days after morphine, the same group of animals conditioned at 20 days received two sessions of startle acclimation, followed by an additional injection of saline or naloxone just before a startle test. Baseline startle magnitude was similar between groups (saline, 37.2 ± 4.4 ; naloxone, 43.2 ± 3.2 ; $F_{1,22}=1.19$, $p=0.29$), but as shown in Fig. 3b, significant startle potentiation was observed following naloxone injection ($F_{1,22}=4.40$, $p=0.048$). Startle is thus significantly potentiated by naloxone

administration for more than 20 days after a single morphine exposure, even though naloxone no longer causes CPA at this time point.

Discussion

Previous research in rodents and primates has demonstrated that chronic opiate exposure can cause long-lasting increases in sensitivity to the administration of opioid receptor antagonists (Celerier et al. 2001; Goldberg and Schuster 1969), as well as persistent changes in rodent behavior (Aston-Jones and Harris 2004; Goeldner et al. 2010) that may parallel the emotional and physiological changes observed in recovering human opiate addicts (Martin and Jasinski 1969; Satel et al. 1993). These changes endure long after the termination of drug exposure, defining a state that has been referred to as either protracted abstinence (Goeldner et al. 2010; Koob and Le Moal 2008; Martin and Jasinski 1969) or protracted withdrawal (Aston-Jones and Harris 2004; Satel et al. 1993). While a single opiate exposure also causes rapid physiological changes that are revealed by antagonist administration (i.e., acute dependence), the persistence of acute dependence has not been thoroughly investigated. The present results represent one of the first demonstrations that at least some facets of acute dependence persist for remarkably long periods of time, after as little as one morphine exposure.

In an initial experiment, we confirmed our previous report (Rothwell et al. 2009) that naloxone administration significantly potentiates acoustic startle 24 h after a single morphine exposure. This effect depended on the dose of naloxone, with a minimum effective dose of 2.5 mg/kg. This naloxone dose dependence is similar to another study where naloxone was administered 2 h after acute morphine exposure (Harris et al. 2004). Importantly, a dose of 2.5 mg/kg naloxone had no effect on startle amplitude in morphine-naïve animals, confirming a number of previous reports from our lab and others (Davis et al. 1988; Glover and Davis 2008; Harris et al. 2004; Hijzen et al. 1995; Rothwell et al. 2009). This demonstrates the specific shift in sensitivity to opioid receptor antagonists (Holtzman 2003; White-Gbadebo and Holtzman 1994) that defines acute dependence.

Although limited clinical evidence suggests opioid receptor antagonists can precipitate signs of withdrawal up to a week after a single opiate administration (Nutt and Jasinski 1974), few preclinical studies have attempted to map the persistence of acute dependence. To investigate this issue, we injected naloxone at progressively later time points after a single morphine exposure. Remarkably, we continued to observe significant startle potentiation up to 80 days after just one morphine exposure. The degree of startle potentiation appeared to be constant across time; we found no evidence for a time-dependent shift in naloxone sensitivity. To our knowledge, this represents the first demonstration that any facet of acute dependence persists for weeks after a single opiate exposure. However, long-lasting effects of one morphine exposure have been reported in other behavioral domains (Rosenfeld and Burks 1977; Vanderschuren et al. 2001).

Startle potentiation is a well-validated measure of anxiety-like behavior across several species (Davis et al. 2010), and we have previously shown that anxiolytic drugs attenuate startle potentiation during either spontaneous or precipitated withdrawal from acute morphine exposure (Harris and Gewirtz 2004; Rothwell et al. 2009). In this respect, our results are consistent with previous reports that various forms of anxiety-like behavior are enhanced in rodents for weeks after chronic exposure to opiates and other drugs (Aston-Jones and Harris 2004; Barr et al. 2010; Valdez et al. 2002; Zhao et al. 2007). Other literature suggests that chronic drug exposure persistently enhances anxiety-like responses to stressful experience, without altering baseline levels of these responses (Blatchford et al. 2005; Jonkman et al. 2008; Rylkova et al. 2009; Smith and Aston-Jones 2008). In rodents,

naloxone administration increases plasma corticosterone levels 24 h after a single morphine exposure (Eisenberg 1982), similar to clinical reports in opiate-dependent human patients (Culpepper-Morgan and Kreek 1997). One day after a single morphine exposure, naloxone also causes immediate early gene induction in the rodent amygdala (Jin et al. 2004)—a key component of brain stress circuits (Koob and Le Moal 2008; Smith and Aston-Jones 2008). These results may indicate that naloxone functions as a form of pharmacological stress (See and Waters 2010), similar to other anxiogenic drugs like yohimbine, which enhances startle magnitude (Stine et al. 2001) and self-reported anxiety (Stine et al. 2002) in opiate-dependent human patients.

Naloxone administration also caused CPA 24 h after a single morphine exposure, consistent with previous reports (Araki et al. 2004; Jin et al. 2004; Parker and Joshi 1998; Rothwell et al. 2009). However, CPA was no longer induced 20 days after a single morphine exposure, in contrast to potentiated acoustic startle as well as the persistence of previously established CPA (Myers and Carlezon 2009; Stinus et al. 2000). This was not due to a lack of naloxone efficacy, since the same group of animals exhibited significant startle potentiation after an additional naloxone injection. We previously reported a similar distinction between the time course of startle potentiation and CPA during spontaneous withdrawal from acute morphine exposure (Rothwell et al. 2009). This suggests CPA may represent a facet of the withdrawal syndrome that is distinct from anxiety, such as dysphoria (Land et al. 2008). Indeed, emerging evidence indicates that anxiety-like behavior and CPA are mediated by distinct neural mechanisms (Bruchas et al. 2009; Land et al. 2008; Sahuque et al. 2006).

The results presented in this study raise important considerations regarding potential mechanisms of naloxone-precipitated withdrawal over short and long time scales. The state of withdrawal precipitated by naloxone in the hours following acute opiate exposure likely reflects competitive displacement of opioid receptor binding by exogenous opiates. While it is possible this mechanism could contribute for at least 24 h after a single opiate exposure (Mullis et al. 1979), it also appears unlikely to explain the effects of naloxone after days or weeks. A second possibility is that naloxone operates by antagonizing increased release of endogenous opioids after acute exposure to an exogenous opiate (Nylander et al. 1995; Olive et al. 1995). This type of upregulation of opioid receptor signaling may represent homeostatic compensation that masks the negative residual of opiate exposure and withdrawal. An alternative form of homeostatic compensation could involve generation of constitutively active opioid receptors (Shoblock and Maidment 2006; 2007). Naloxone exhibits pharmacological properties of an inverse agonist, capable of reversing constitutive activation of opioid receptors (Sadée et al. 2005), and potentially revealing an otherwise occult state of dependence.

Regardless of the specific mechanisms involved, it is remarkable that a single morphine exposure causes such enduring changes in opioid receptor signaling and behavior. The conservation of the startle reflex across species raises the possibility that similar methodology could be used to study protracted manifestations of acute dependence in humans (Krystal et al. 1997; Stine et al. 2001). Our results suggest these manifestations may be specifically related to anxiety, though other components of withdrawal may be engaged after more extensive regimens of opiate exposure (Celerier et al. 2001; Goeldner et al. 2010). The utility of acoustic startle for studying the mechanisms of fear learning (Davis et al. 2010) should also facilitate comparative studies of the overlapping neural circuitry underlying states of learned fear, anxiety, and drug withdrawal (Koob and Le Moal 2008; Smith and Aston-Jones 2008). Further study of persistent adaptations in these circuits caused by acute opiate exposure should facilitate our understanding of the development and escalating severity of drug dependence and addiction.

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Fig. 1. Dose-dependent potentiation of acoustic startle by naloxone, 24 h after a single morphine exposure. Each *bar* represents a separate group of six rats; 0 mg/kg indicates injection of saline vehicle. Significant linear effect of naloxone dose (*asterisk*) ($p < 0.05$, one-way ANOVA)

Fig. 2.

Naloxone administration potentiates acoustic startle for at least 80 days after a single morphine exposure. Data for 1 day (D1) are reproduced from Fig. 1; *every other bar* represents a separate group of 6 (D5), 12–13 (D10), 6 (D20), or 11–12 (D80). Significant difference between groups (*asterisk*) ($p < 0.05$, one-way ANOVA)

Fig. 3.

Naloxone causes CPA 1 day but not 20 days after a single morphine injection. **a** Percent time spent in the environment associated with naloxone before (baseline) and after (test) conditioning. Each *pair of bars* represents a separate group of 16 (D1) or 24 (D20). Significant difference between baseline and test (*asterisk*) ($p < 0.05$, one-way repeated measures ANOVA). **b** Percent change in acoustic startle following an additional injection of saline ($n = 12$) or naloxone ($n = 12$) in the same animals tested for CPA 28 days post-morphine. Significant difference between groups (*asterisk*) ($p < 0.05$, one-way ANOVA)

Table 1

Baseline startle values for groups tested with naloxone at different time points after a single morphine exposure

Time point (days)	Saline	Naloxone	Statistics
1	29.6±3.1	25.9±3.3	$F_{1,10}<1$
5	41.1±4.3	30.4±4.2	$F_{1,10}=2.96, p=0.11$
10	44.2±4.4	39.9±3.0	$F_{1,23}<1$
20	42.2±7.5	39.5±4.5	$F_{1,10}<1$
80	60.2±5.9	49.2±5.4	$F_{1,21}=1.90, p=0.18$