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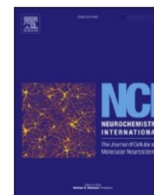

Ghrelin receptor antagonism of morphine-induced conditioned place preference and behavioral and accumbens dopaminergic sensitization in rats

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a b s t r a c t

An increasing number of studies over the past few years have demonstrated ghrelin's role in alcohol, cocaine and nicotine abuse. However, the role of ghrelin in opioid effects has rarely been examined. Recently we substantiated in rats that ghrelin growth hormone secretagogue receptors (GHS-R1A) appear to be involved in acute opioideinduced changes in the mesolimbic dopaminergic system associated with the reward processing. The aim of the present study was to ascertain whether a ghrelin antagonist (JMV2959) was able to inhibit morphine-induced biased conditioned place preference and challenge-morphine-induced accumbens dopaminergic sensitization and behavioral sensitization in adult male rats. In the place preference model, the rats were conditioned for 8 days with morphine (10 mg/kg s.c.). On the experimental day, JMV2959 (3 and 6 mg/kg i.p.) or saline were administered before testing. We used in vivo microdialysis to determine changes of dopamine and its metabolites in the nucleus accumbens in rats following challenge-morphine dose (5 mg/kg s.c.) with or without JMV2959 (3 and 6 mg/kg i.p.) pretreatment, administered on the 12th day of spontaneous abstinence from morphine repeated treatment (5 days, 10e40 mg/kg). Induced behavioral changes were simultaneously monitored. Pretreatment with JMV2959 significantly and dose dependently reduced the morphine-induced conditioned place preference and significantly and dose dependently reduced the challenge-morphine-induced dopaminergic sensitization and affected concentration of by-products associated with dopamine metabolism in the nucleus accumbens. JMV2959 pretreatment also significantly reduced challenge-morphine-induced behavioral sensitization. Our present data suggest that GHS-R1A antagonists deserve to be further investigated as a novel treatment strategy for opioid addiction.

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1. Introduction

Gut-brain peptide hormone ghrelin (Kojima et al., 1999) has been recently shown to play a crucial role in food reward (Egecioglu et al., 2010) as well as reward, motivation and intake of alcohol and reward of cocaine, amphetamine, and nicotine; for review see (Engel and Jerlhag, 2014; Panagopoulos and Ralevski, 2014). In addition to hypothalamus, the central ghrelin secretagogue receptors (GHSR1A[1]) are expressed in important reward related areas including striatum, nucleus accumbens (NAC[2]), amygdala, prefrontal cortex, hippocampus, and ventral tegmental area (VTA[3]) (Abizaid et al., 2006; Ferrini et al., 2009; Howard et al., 1996; Landgren et al., 2011; Naleid et al., 2005; Quarta et al., 2009; Skibicka et al., 2011; Zigman et al., 2006). Central GHS-R1A receptors are frequently co-localized with dopaminergic and cholinergic receptors (Ferrini et al., 2009; Guan et al., 1997) and it is

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[1] Growth hormone secretagogue receptor A1 for ghrelin.
[2] Nucleus accumbens.
[3] Ventral tegmental area.

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supposed that midbrain functional interactions between these receptors amplify the dopaminergic signaling in the VTA neurons and stimulate the overflow of dopamine in the NAC (Jerlhag et al., 2006, 2011).

Increased extracellular dopamine levels in the NAC, mainly in the shell substructure, have been associated with the acute rewarding/reinforcing effects of addictive drugs, including opioids (De Vries and Shippenberg, 2002; Di Chiara, 2002; Di Chiara and Imperato, 1988; Hyman et al., 2006; Koob, 1992; Koob and Volkow, 2010; Leone et al., 1991; Pothos et al., 1991). Opioids activate the mesolimbic dopaminergic system indirectly (predominantly through m opioid receptors in the VTA and NAC) considering several possible modulatory mechanisms with presumed participation of different mediator/neural systems, such as GABA, acetylcholine, glutamate, endocannabinoids, and also probably ghrelin and others (Fiserova et al., 1999; Koob and Volkow, 2010; Leone et al., 1991; Pothos et al., 1991; Sustkova-Fiserova et al., 2014, 2016; Vaccarino et al., 1985; Wise and Rompre, 1989). Beside other adaptive changes, repeated intermittent exposures to addictive drugs typically lead to drug sensitization, behavioral sensitization and differentially sensitized mesolimbic dopaminergic system, depending on the appropriate conditions of drug manipulations. Concerning the NAC, higher stimulatory impact of drug reward combined with drug-conditioned stimuli was observed in the shell substructure compared to core dopamine transmission. On the contrary, non-associative drug sensitization increased the stimulatory impact of drugs, including opioids, on NAC core dopamine while rather reducing that on the NAC shell (Berridge and Robinson, 1998; Cadoni and Di Chiara, 1999; Di Chiara, 2002; Di Chiara et al., 2004; Charmchi et al., 2016; Koob and Volkow, 2010; Robinson and Berridge, 2003; Vanderschuren and Kalivas, 2000). Hypersensitivity of mesolimbic dopaminergic neurons during a protracted abstinence may underlie the long-term expression of behavioral sensitization to drugs of abuse as well as the reinstatement of compulsive drug-seeking behavior (Robinson and Berridge, 1993b; Vanderschuren and Kalivas, 2000). Behavioral sensitization can be used within certain limits for investigating the incentive motivation of underlying drug-seeking behavior. Mesolimbic dopamine system mediates both the incentive-motivational and sensitizing properties of opioids and other drugs of abuse (De Vries and Shippenberg, 2002; De Vries et al., 1998; Robinson and Berridge, 2003; Steketee and Kalivas, 2011; Vanderschuren and Kalivas, 2000). As such, manipulations that attenuate drug/opioid accumbens dopaminergic and behavioral sensitization might be of value for the conceptualization and perhaps treatment of addiction (Steketee and Kalivas, 2011; Vanderschuren and Kalivas, 2000).

Current literature implicating ghrelin in opioid use disorders is still limited and inconclusive (D'Cunha et al., 2013; Engel et al., 2015; Maric et al., 2012; Sustkova-Fiserova et al., 2014, 2016). The only opioid/heroin self-administration study (Maric et al., 2012) showed that ghrelin (i.c.v.) was able to increase heroin intake, yet pretreatment with the peptide GHS-R1A antagonist (i.c.v.) did not influence heroin self-administration. However, in our previous study (Sustkova-Fiserova et al., 2014), we demonstrated in rats that pretreatment with the GHS-R1A antagonist JMV2959, a non-peptidic triazole substance (Moulin et al., 2007), significantly and dose-dependently attenuated acute morphine-induced dopamine release in the NAC shell as well as stereotypical behaviors and locomotion. These results have been recently confirmed in mice (Engel et al., 2015).

A role of ghrelin in the opioid-induced accumbens dopamine sensitization, which is believed to be important in opioid reinforcement and dependence, has not been studied so far. The aim of the first part of our study was to ascertain whether ghrelin antagonism was able to inhibit expression/manifestation of repeated morphine-induced sensitization in rats. More precisely, we investigated the influence of ghrelin antagonist (JMV2959) on sensitized accumbens dopamine release and concomitant behavioral sensitization revealed by morphine challenge administered on the 12th day of abstinence following 5 days of morphine treatment. In contrast to our previous study (Sustkova-Fiserova et al., 2014), when the dopaminergic changes were observed in the NAC shell after acute morphine dose administration, here we choose coordinates including also the NAC core substructure, because as mentioned above, the non-associated opioid sensitization has been linked with the dopaminergic hypersensitivity preferentially in the NAC core (Cadoni and Di Chiara, 1999; Di Chiara, 2002; Spanagel et al., 1993). In order to get a more complete picture, we also monitored the dopaminergic metabolism in the NAC. Our previous study (Sustkova-Fiserova et al., 2016) has indicated an important participation of ghrelin in challenge morphine-induced sensitized stereotypical behaviors and locomotion. Herein the effects of ghrelin antagonism on morphine-induced behavioral sensitization were characterized as a complex, when calculated in percentage of time spent by the animals in appropriate behavioral categories.

Opioid-induced conditioned place preference (CPP), as with other addictive drugs, is closely associated with the opioid reinforcing properties, which are considered to constitute a part of the addiction process (Bardo and Bevins, 2000; Bardo et al., 1995). Thus, to further establish the role of ghrelin in the rewarding effects of opioids, the aim of the second part of this study was to test, whether ghrelin antagonism would attenuate the expression of morphine-induced CPP in rats, which has been recently suggested in mice (Engel et al., 2015).

The ability of opioids to induce accumbens dopamine sensitization, behavioral sensitization and conditioned place preference are closely associated with their reinforcing properties, which are considered to participate crucially in the opioid addiction process. Thus our presented study intends to further test and substantiate the idea, that GHS-R1A antagonism might be considered as potential novel treatment strategy of opioid addiction.

2. Materials and methods

2.1. Animals

Male Wistar rats (Velaz, Anlab Czech Republic), approx. 8 weeks old, weighing 200e250 g were used. The animals were given free access to water and food, and were housed in polycarbonate cages with constant humidity (50e60%), room temperature (22e24 °C), and a 12-h light/dark cycle, for at least 7 days before the experiments, which were performed from 8 a.m. to 3 p.m. Groups of 6 rats were used for each treatment in the microdialysis experiments, groups of 14e15 rats were used in the conditioned place preference (CPP) method and groups of 9 rats were used in the "open field" test. Procedures involving animals and animal care were conducted in compliance with international laws; protocols respected the Guidelines of the European Union Council (86/609/EU) and EU Directive (2010/63/EU) and followed the instructions of the National Committee for the Care and Use of Laboratory Animals. Experiments were approved by the Expert Committee for Protection of Experimental Animals of the Third Faculty of Medicine, Charles University in Prague and were performed in accordance with the Animal Protection Act of the Czech Republic (No. 246/1992 Sb).

2.2. Drugs and chemicals

Morphine hydrochloride was purchased from Dr. Kulich Pharma (CR). JMV2959 (1,2,4-triazole derivate), which has been proved to be an GHS-R1A antagonist (Moulin et al., 2007), was kindly

provided by Anton Bespalov, AbbVie, Germany. Both substances were dissolved in saline and saline was used as a placebo. Morphine (5 or 10 mg/kg) was administered subcutaneously (s.c.) in volumes of 0.1 ml/100 g of body weight. The selected doses of JMV2959 (3 and 6 mg/kg) were determined based on our previous studies in Wistar rats (Sustkova-Fiserova et al., 2014, 2016) and the literature (Clifford et al., 2012; Jerlhag et al., 2010). The lower JMV2959 dose (3 mg/kg) had no effect on the rat behavior. Similarly to our previous studies, the higher JMV2959 dose (6 mg/kg) caused temporary behavioral changes (stretching elike movements) in less than 40% of the treated rats, which were fully eliminated with sound or touch and spontaneously disappeared within 20 min after administration. JMV2959 was administered intraperitoneally (i.p.) 0.1 ml/100 g of body weight and always 20 min prior the CPP testing or prior to morphine or saline injections. All reagents were analytical grade.

2.3. *In vivo microdialysis: assay of dopamine and its metabolites*

To test the ghrelin antagonism effects on the challenge morphine-induced accumbens dopaminergic sensitization and concomitant behavioral sensitization, morphine was applied once a day for 5 consecutive days in increasing doses (10, 20, 20, 40, 40 mg/kg s.c.), followed by period of abstinence. Animals were housed in twos in cages of the same size as the experimental once during the application days. On the 10th day of abstinence the rats were implanted with the microdialysis guide cannula into the NAC, and on the 12th day of abstinence, when the in vivo microdialysis was performed, morphine challenge dose (5 mg/kg s.c.) or saline (s.c.) was applied with pretreatment of JMV2959 (3 or 6 mg/kg i.p.) or saline (i.p.). We have chosen coordinates including also the NAC core substructure, because opioid sensitization has been connected with the dopaminergic hypersensitivity preferentially in the NAC core (Cadoni and Di Chiara, 1999; Di Chiara, 2002). Simultaneously with dialysis, the same animals were monitored for morphine-induced behavioral changes. Treatment groups sensitized with chronic morphine during the dialysis were as follows: saline þchallenge saline; saline þchallenge morphine 5 mg/kg; JMV2959 3 mg/kg þ challenge saline; JMV2959 6 mg/kg þ challenge saline; JMV2959 3 mg/kg þ challenge morphine 5 mg/kg; JMV2959 6 mg/kgþchallenge morphine 5 mg/kg. One group of rats was treated with chronic saline (0.1 ml/100 g of body weight) instead of chronic morphine and morphine was administered only during microdialysis: salineþmorphine. The dialysis samples were collected at 20 min intervals for a total of 260 min. Dialysates were analyzed for the concentration of dopamine and its metabolites (3-methoxytyramine (3-MT), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA)) using high-sensitivity liquid chromatography combined with mass spectrometry (HPLC-MS).

2.3.1. *Surgery*

The method is described in detail in Sustkova-Fiserova et al., (2014, 2016). On the 10th day of abstinence, under ketamine e xylazine anaesthesia (ketamine 100 mg/kg i.p., Narketan, Vetoquinol; xylazine 10 mg/kg i.p., Xylapan, Vetoquinol), rats were implanted with a disposable dialysis guide cannula (MAB4 probes, Agnthos, Sweden) using a stereotaxic instrument (Stoelting Co) into the nucleus accumbens core/shell (Aþ1.7 mm and L: ±1.5 mm from bregma and V: 6.1 mm from occipital bone) (Paxinos and Watson, 2006) and secured to the skull with dental cement and an anchoring screw. The guide was randomly alternated on the left and right side. After surgery, the rats were kept in individual cages. After completion of the microdialysis experiments, the placement of the dialysis probe was verified histologically (Fig. 1). Only animals with correct probe placement were used for subsequent statistical analysis.

2.3.2. *Microdialysis and chemical analysis assay*

In accordance with Sustkova-Fiserova (Sustkova-Fiserova et al., 2014, 2016), 48 h after implantation, the probe (MAB4, 2 mm active cuprophane membrane, Agnthos, Sweden) was inserted into the guide cannula and artificial cerebrospinal fluid (Ringer's solution; 147 mM NaCl, 2. 2 mM CaCl2 and 4.0 mM KCl; adjusted to pH 7.0) was flushed through the probe at a constant rate of 2.0 ml/min (Univentor 864 Syringe Pump, Agnthos, Sweden). After a minimum 40 min of habituation (the dialysate was discarded), 20 ml samples were collected at 20-min intervals in small polyethylene test tubes containing 7 ml HCl 0.1 mM, to prevent catecholamine hydrolysis. The other 20-ml part of each interval dialysate was used for detection of other neurotransmitters (in preparation). After 3 consecutive baseline samples, rats were injected with saline or JMV2959 (i.p.), which was followed (20 min later) by morphine or saline (s.c.) injection (in separate experiments). Samples were collected for 3 h following injection of morphine or saline. Immediately following collection, the samples were frozen at −70 ° C. The amount of dopamine and its metabolites (3-MT, DOPAC and HVA) in the dialysate were quantified using HPLC-MS. The used HPLC-MS arrangement is described in detail in Sustkova-Fiserova et al., 2014 and Syslova et al., 2011. Thus, here only brief explanation: after freeze-drying (lyophilization in the freeze dryer; Labconco Free Zone, USA) to concentrate the substances from the microdialysates, the content of dopamine and its metabolites was determined using liquid chromatography combined with electrospray ionization tandem mass spectrometry (LCeESI-MS/MS; consisted of a chromatograph Accela 1250 (Thermo Scientific, USA), autosampler Accela (Thermo Scientific, USA) and a TSQ Vantage mass spectrometer (Thermo Scientific, USA). The data were acquired and processed using Xcalibur 2.1.0 software (Thermo Scientific, USA).

2.4. *Behavior monitoring during in vivo microdialysis*

Behavior was studied simultaneously, in the same animals, while microdialysis measurements were being performed (as described previously (Sustkova-Fiserova et al., 2014, 2016)). The following behavioral categories were distinguished: immobility (sedation, eyes closed, akinesia, and reduced responsiveness to environmental cues), catalepsy (frozen postures, exophthalmos, and trunk rigidity), locomotion (non-stereotyped activity, sniffing, grooming, rearing, and walking), stereotyped activity (confined gnawing, licking, and stereotypical sniffing), and other symptoms (stretch-like behavior) similar to (Acquas and Di Chiara, 1992; Fiserova et al., 1999; Rada et al., 1991; Sustkova-Fiserova et al., 2014). Behavioral categories were scored every 20-min (at each microdialysis interval) by an observer who was unaware of the treatment each rat had received. The percentage of time spent by the animal in each behavioral category was calculated for each 20-min interval. Behavioral changes were monitored during the entire dialysis period (60 min baseline, 20 min pre-treatment and 3 h following morphine or saline injection).

2.5. *Conditioned place preference*

To evaluate the effects of GHS-R1A on the rewarding properties of morphine, biased conditioned place preference (CPP) method was performed in rats, based on Sanchis-Segura and Spanagel (2006), Jerlhag et al. (2010) etc. A three-compartment chamber of CPP apparatus, with distinct visual and tactile cues in the outer compartments, was used. One outer compartment was defined by

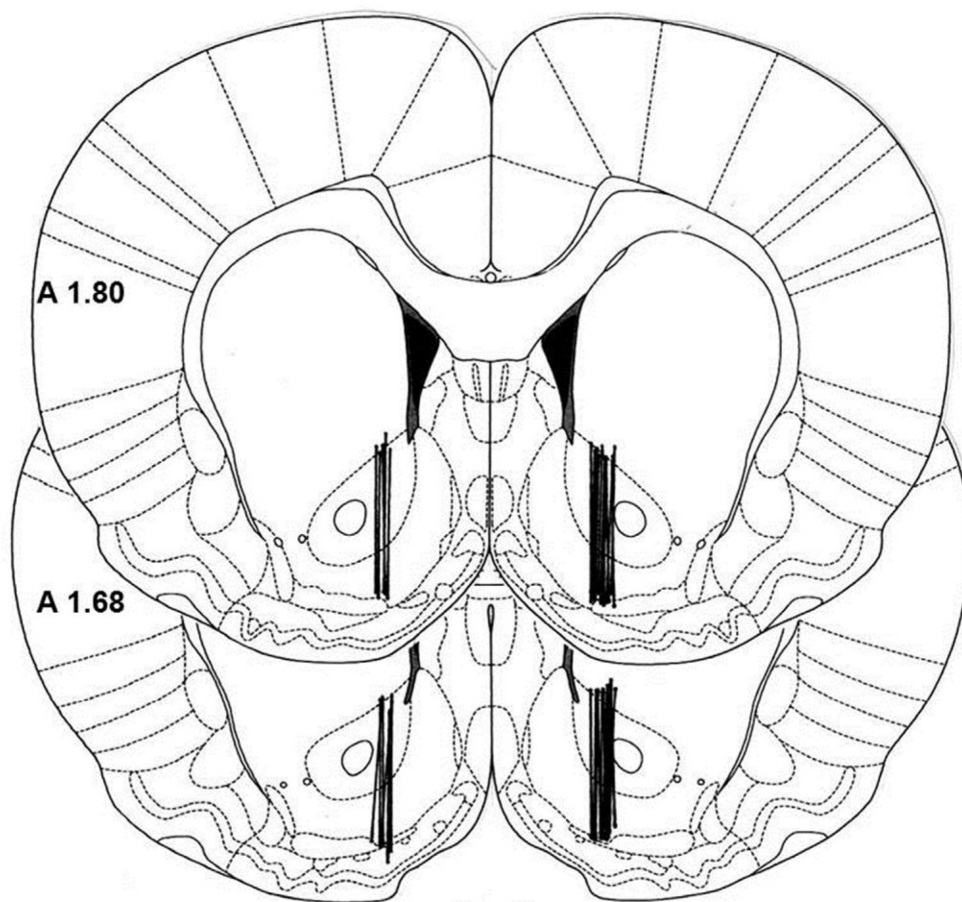

Fig. 1. Location of dialysis probes within the nucleus accumbens core/shell. Schematic locations of probe tips in rats which were included in analyses of accumbens neurotransmitter concentrations (the solid lines indicate the dialyzing portions) as described in the atlas of Paxinos and Watson (Paxinos and Watson, 2006). On the left, for each section, the distance from bregma (in mm) is indicated.

wide horizontal black- and white-striped walls and by a coarse grid floor, whereas the other had much finer grid floor and narrow vertical black- and white-striped walls. The center compartment had no special characteristics, and the gates between the compartments could be opened to allow an animal to pass freely between them. All compartments were illuminated by 45 lux. The procedure consisted of pre-conditioning (day 1), conditioning (days 2e9), and post-conditioning (day 10). On day 1 (pre-conditioning), each rat was i.p. injected with saline 20 min prior testing, then placed in the center compartment with both gates open, and initial place preference was determined during 20 min in order to determine which of the two compartments could be labeled spontaneously “least preferred” for each rat. Conditioning was done using a repetitious procedure in which morphine was paired to the least preferred compartment. Each rat received a total of two i.p. injections per day where in a balanced design, morphine (10 mg/kg s.c.) was administered in the morning and saline conditioning in the afternoon or vice versa. After drug injection, the rat was placed in the appropriate outer compartment (for 40 min, with the gate closed). On day 10 (post-conditioning test session), the rats were placed in the center compartment (with the gates open) and were thereafter given free access to both compartments for 20 min. 20min prior to the test session, each rat was acutely injected with JMV2959 (3 or 6 mg/kg i.p.) or saline (i.p). It has been proved that application of vehicle/saline does not induce any CPP conditioning. It has been also described that JMV2959 has no effect per se on CPP (Jerlhag et al., 2009). Therefore these experiments were not included. CPP was calculated as the difference in percentage of total time spent in the morphine-paired (i.e., least preferred) compartment during the post-conditioning and the pre-conditioning session.

2.6. *Additional tests of JMV2959 effects and GHS-R1A involvement in morphine mechanisms*

2.6.1. *Open field (Ethovision)*

Open field test was used in order to further explore the influence of JMV2959 on rat behavior. Twenty five minutes after administration of JMV2959 (1 or 3 or 6 mg/kg i.p.) or saline, the rat was placed into a standard square rat open field arena (Ugo Basile) and the explorative locomotor behavior was monitored for 20min. At the end of the test, the rats were removed from the arena and the floor was cleaned and dried. The rats'activity was recorded by a fixed digital camera located above the arena and the video feed was transferred to a PC-based tracking system (Noldus EthoVision, Noldus Information Technology, The Netherlands) that extracted and stored x-y coordinates. The distance traveled, behavior velocity and time spent in the central zone were calculated within two consequent 10min intervals and total 20min interval; the tracking rate was 10 frames per second and the space resolution was about 0.8 cm. The effects of JMV2959 groups were compared with saline group. In all groups N ¼ 9.

2.6.2. *In vivo microdialysis acute experiment*

The details of the microdialysis method are described above (section 2.3.1. e 2.) The acute effects of morphine in rats, after pretreatment with JMV2959 or saline, were monitored using in vivo microdialysis of the nucleus accumbens (NAC shell/core). To verify the involvement of ghrelin antagonism in the tested mechanisms, in one rat group, ghrelin was administered together with JMV2959 intraperitoneally separately in the opposite sites 20 min before morphine. Thus, treatment groups in the acute experiment were as follows: saline þ saline, saline þ morphine 10 mg/kg, JMV2959 3 mg/kg þ morphine 10 mg/kg, JMV2959 3 mg/kg together with ghrelin 40 mg/kg þ morphine 10 mg/kg, JMV2959 3 mg/kgþsaline, ghrelin 40 mg/kgþsaline. The dialysis samples were collected at 20 min intervals for a total of 260 min and dialysates were analyzed for the concentration of dopamine using HPLC-MS, as it was previously described above (section 2.3.1. e 2.).

2.7. *Statistical analysis*

Raw data for dopamine and its metabolites (expressed as picogram per milliliter per sample, not corrected for probe recovery) were transformed into a percentage of baseline levels (mean of three values prior to pretreatment). Changes in behavioral parameters during the 20-min intervals, were also analyzed. Time course neurochemical and behavioral data were statistically analyzed using SigmaStat 3.5, Systat Software, Inc., USA. For statistical differences between the appropriate treatment groups (JMV2959 þmorphine), (saline þ morphine), and (saline þ saline) in experiments relative to time-related changes in the course of the in vivo microdialysis study, a two-way analysis of variance for repeated measures (ANOVA RM analysis) followed by Bonferroni corrected linear contrasts test was used. In this ANOVA analysis, the group of rats was entered as the between-group factor and the time-points as repeated within-subject measures (to compare all treatments to baseline mean; 20-min intervals over 200 min of post-treatment). Place preference scores (CPP) were computed as the difference in percentage (%) of total time spent in the morphine-paired (i.e., least preferred) compartment during the post-conditioning and the pre-conditioning session. The differ- ences between groups in the CPP were evaluated by a one-way ANOVA followed by Holm-Shidak post-hoc test. The data ob- tained from open field (Ethovision) test, comparison of three JMV2959 treated groups against saline treated group, were calcu- lated within two consequent 10min intervals using two-way ANOVA RM analysis and within total 20min interval using one- way ANOVA. All statistical tests were evaluated as two-sided at a significance level of 0.05 (P values of <0.05, <0.01 and < 0.001 defined statistical significance). Results are presented as the mean ± SEM.

3. Results

3.1. *Microdialysis and chemical analysis assay*

3.1.1. *The challenge morphine-induced accumbens dopamine release and dopamine metabolism in rats sensitized to morphine*

As expected and as illustrated in Fig. 2a, systemic administration of 5 mg/kg challenge morphine dose on the 12th day of abstinence following 5 days of morphine treatment induced significantly higher extracellular concentration of dopamine in the rat NAC core-shell in comparison with the acute morphine effects, when morphine was administered following chronic saline treatment. Thus, accumbens dopamine sensitization was substantiated. The two-way ANOVA for repeated measures (RM) followed by Bonferroni's test revealed a significant group effect: saline þ challenge morphine 5 mg/kg vs. saline þ acute morphine group ($F_{1,10}$ ¼ 10.45, P < 0.01) and time ($F_{10,100}$ ¼ 156.33, P < 0.001); time course of accumbens dopamine changes after challenge/acute morphine administration differed significantly between the two rat groups (time × group interaction $F_{10,100}$ ¼ 3.19, P ¼ 0.001). Both, acute and challenge morphine induced dopamine increases in comparison to baseline were also significant (P < 0.001) with the maximum effects occurring at 60 min post-administration (168% and 194% of baseline levels respectively). Dopamine baseline levels did not differ significantly between animals in both acute and longer-term morphine administration.

The dopamine metabolites 3-MT and DOPAC accumbens extracellular concentrations (Fig. 2b and c) did not differ significantly between acute and sensitized experiments after morphine administration. Challenge morphine-induced accumbens HVA concentration (Fig. 2d) was temporarily significantly higher in rats sensitized to morphine in comparison to the acute morphine effects (saline þchallenge morphine 5 mg/kg vs. salineþ acute morphine: effect of group $F_{1,10}$ ¼ 6.17, P < 0.05; effect of time $F_{10,100}$ ¼ 54.75, P < 0.001; time× group interaction $F_{10,100}$ ¼ 3.13, P¼0.001). All dopamine metabolites baseline levels did not differ significantly between the acute and longer-term morphine treated groups.

3.1.2. *The effects of GHS-R1A antagonist on the challenge morphine-induced accumbens dopaminergic sensitization*

The effects of ghrelin antagonism on changes in accumbens dopamine release induced by a 5 mg/kg morphine challenge applied on the 12th day of abstinence following 5 days of morphine treatment are illustrated in Fig. 3a. Dopamine baseline levels did not differ significantly among animals. As mentioned above, 5 mg/kg challenge morphine induced increased dopamine release in the NAC of rats sensitized to morphine (saline þ challenge morphine 5 mg/kg vs. salineþ challenge saline: effect of group $F_{1,10}$¼ 358.28, P < 0.001; effect of time $F_{10,100}$¼ 76.22, P < 0.001; time× group interaction $F_{10,100}$ ¼ 78.63, P < 0.001).

Both, 3 and 6 mg/kg JMV2959 pretreatments significantly and dose-dependently reduced the challenge morphine-induced accumbens dopamine increase (JMV2959 3 mg/kgþ challenge morphine 5 mg/kg vs. salineþchallenge morphine 5 mg/kg: effect of group $F_{1,10}$ ¼ 125.35, P < 0.001; effect of time $F_{10,100}$ ¼ 133.48, P < 0.001; time ×group interaction $F_{10,100}$ ¼ 23.28, P < 0.001; JMV2959 6 mg/kg þ challenge morphine 5 mg/kg vs. saline þ challenge morphine 5 mg/kg: effect of group $F_{1,10}$ ¼ 222.92, P < 0.001; effect of time $F_{10,100}$ ¼ 128.60, P < 0.001; time ×group interaction $F_{10,100}$ ¼ 42.26, P < 0.001). However, within both JMV2959 pretreated groups the observed challenge morphine-induced dopamine increases in comparison to baselines still remained significant (P < 0.001) with maximum effect occurring at 60 min post-administration: 92% of baseline in case of 3 mg/kg and 41% in 6 mg/kg JMV2959. A single challenge 3 mg/kg dose of JMV2959 had no effect on accumbens dopamine. The higher 6 mg/kg dose of JMV2959 induced a minor temporarily significant decrease in accumbens dopamine (JMV2959 6 mg/kgþ challenge saline vs. saline þ challenge saline: effect of group $F_{1,10}$¼ 6.28, P < 0.05; effect of time $F_{10,100}$¼ 5.95, P < 0.001; time× group interaction $F_{10,100}$ ¼ 3.71, P < 0.001). Application of saline had no effect on accumbens dopamine.

3.1.3. *The effects of GHS-R1A antagonist on the challenge morphine-induced changes in accumbens dopamine metabolites*

The influence of ghrelin antagonism on changes in extracellular accumbens concentrations of dopamine metabolites induced by a 5 mg/kg morphine challenge applied on the 12th day of abstinence following 5 days of morphine treatment are illustrated in Fig. 3b,c,d.

For 3-MT (Fig. 3b), the challenge 5 mg/kg morphine induced

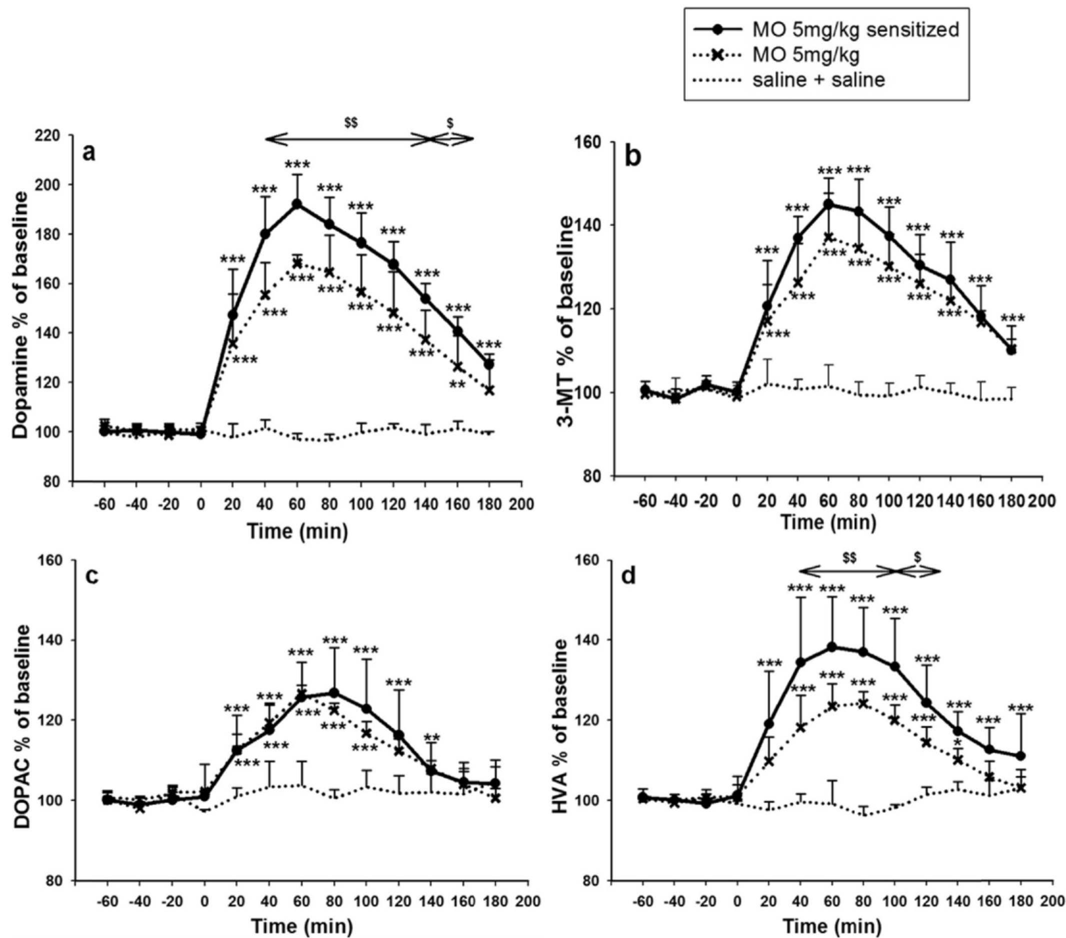

Fig. 2. Challenge morphine-induced accumbens dopamine release and dopamine metabolism in rats sensitized to morphine. 5 mg/kg acute/challenge morphine was injected s.c. 20 min after i.p. saline (0.1 ml/100 g of body weight) to rats sensitized to morphine or following chronic saline treatment (n ¼ 6; means ± SEM). Changes in accumbens dopamine concentrations are illustrated in the graph a, changes in 3-MT in b, DOPAC in c and HVA in the graph d. The effects are illustrated as follows: saline þ challenge morphine in rats sensitized to morphine (filled circle), saline þ morphine acute (dotting with crosses), saline þ saline (dotting). Differences between treatments and baseline mean within a group are expressed as ***P < 0.001, **P < 0.01, *P < 0.05. Differences between groups are expressed as $$ P < 0.01, $ P < 0.05.

significant increase with a maximum 60 min post-morphine injection (145% of baseline) (saline þ challenge morphine 5 mg/kg vs. saline þ challenge saline: effect of group F1,10 ¼ 135.31, P < 0.001; effect of time F10,110 ¼ 53.39, P < 0.001; time × group interaction F10,100 ¼ 43.22, P < 0.001). Pretreatment with 3 as well as 6 mg/kg JMV2959 significantly and practically coincidently attenuated the challenge morphine-induced 3-MT accumbens increase (JMV2959 3 mg/kg þ challenge morphine 5 mg/kg vs. saline þ challenge morphine 5 mg/kg: effect of group F1,10 ¼ 24.84, P ¼ < 0.001; effect of time F10,100 ¼ 114.22, P < 0.001; time × group interaction F10,100 ¼ 10.54, P < 0.001; JMV2959 6 mg/kg þ challenge morphine 5 mg/kg vs. saline þ challenge morphine 5 mg/kg: effect of group F1,10 ¼ 17.78, P ¼ < 0.001; effect of time F10,100 ¼ 82.01, P < 0.001; time × group interaction F10,100 ¼ 9.48, P < 0.001). The maximum morphine-induced 3-MT increase occurred 60 min post-administration following 3 mg/kg JMV2959 pretreatment and was postponed to 100 min post-administration following 6 mg/kg JMV2959 and dropped to 127% and 124% respectively. The single challenge doses 3 as well as 6 mg/kg JMV2959 did not change the concentration of 3-MT, and the same was true for challenge saline.

The effects of GHS-R1A antagonist on accumbens challenge morphine-induced DOPAC formation is illustrated in Fig. 3c. Challenge 5 mg/kg morphine-induced significant DOPAC increase in the NAC with maximum 126% of baseline 80 min post-morphine injection (saline þ challenge morphine 5 mg/kg vs. saline þ challenge saline: effect of group F1,10 ¼ 18.31, P ¼ < 0.001; effect of time F10,100 ¼ 18.87, P < 0.001; time × group interaction F10,100 ¼ 6.10, P < 0.001). Pretreatment with 3 as well as 6 mg/kg JMV2959 slightly, only temporarily significantly augmented the challenge morphine-induced DOPAC increase in the NAC to maximum 131% of baseline 60 min post-morphine injection (JMV2959 3 mg/kg þ challenge morphine 5 mg/kg vs. saline þ challenge morphine 5 mg/kg: effect of group n.s.; effect of time F10,100 ¼ 53.59, P < 0.001; time × group interaction F10,100 ¼ 2.96, P < 0.05; JMV2959 6 mg/kg þ challenge morphine 5 mg/kg vs. saline þ challenge morphine 5 mg/kg: effect of group n.s.; effect of time F10,100 ¼ 66.08, P < 0.001; time × group interaction F10,100 ¼ 4.01, P < 0.05). The single challenge dose 3 mg/kg JMV2959 had no effect on accumbens DOPAC. The higher dose 6 mg/kg JMV2959 slightly (temporarily significantly) increased the accumbens DOPAC, maximum 108% of baseline, 60 min postinjection (JMV2959 6 mg/kg þ challenge saline vs. saline þ challenge saline: effect of group n.s.; effect of time F10,100 ¼ 6.63, P < 0.001; time × group interaction F10,100 ¼ 4.20, P < 0.001). Application of challenge saline had no effect on DOPAC.

The effects of GHS-R1A antagonist on challenge morphine-induced accumbens HVA increase is illustrated in Fig. 3d.

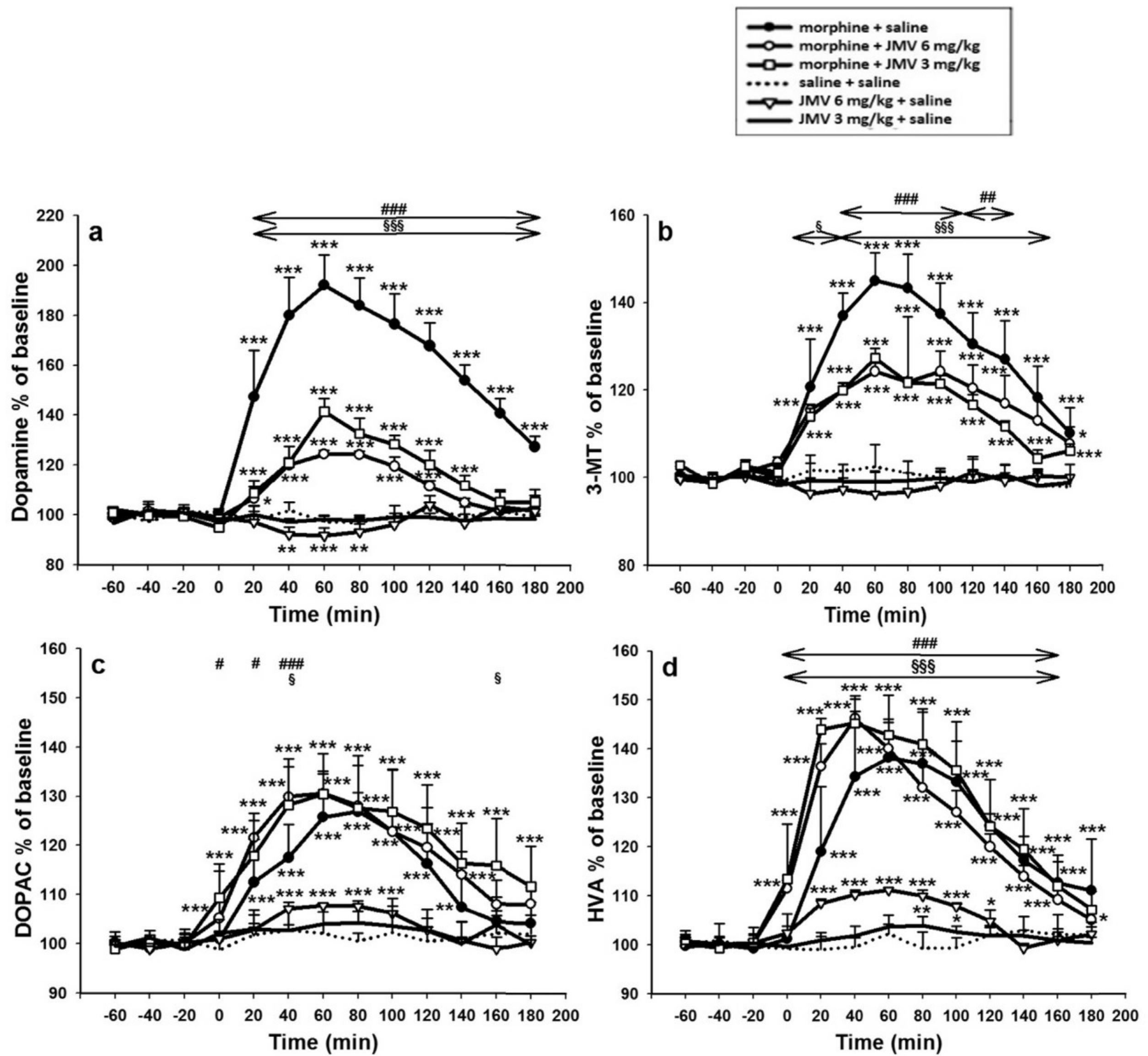

Fig. 3. Effects of ghrelin receptor GHS-R1A antagonist JMV2959 on challenge morphine-induced dopamine and its metabolites extracellular changes in the rat NAC. JMV2959 was always given i.p. 20 min before morphine/saline s.c. injection (n ¼ 6; means ± SEM). Changes in accumbens dopamine concentrations are illustrated in the graph a, changes in 3-MT in b, DOPAC in c and HVA in the graph d. The effects are illustrated as follows: saline þ challenge 5 mg/kg morphine (filled circle), 6 mg/kg JMV2959 þ challenge 5 mg/kg morphine (open circle), 3 mg/kg JMV2959 þ challenge morphine 5 mg (open square), 3 mg/kg JMV2959 þ challenge saline (continuous line), 6 mg/kg JMV2959 þ challenge saline (open triangle), saline þ challenge saline (dotting). Differences between treatments and baseline mean within a group are expressed as ***P < 0.001, **P < 0.01, *P < 0.05. Differences between groups saline þ challenge morphine versus 6 mg/kg JMV2959 þ challenge morphine or saline þ challenge morphine versus 3 mg/kg JMV2959 þ challenge morphine effects are expressed as ###P < 0.001, ##P < 0.01, #P < 0.05 or xxxP < 0.001, xP < 0.05 respectively.

Challenge 5 mg/kg morphine induced significant HVA increase in the NAC with maximum 138% of baseline 60 min post-morphine injection (saline þ challenge morphine 5 mg/kg vs. saline þchallenge saline: effect of group F1,10¼33.22, P < 0.001; effect of time F10,100¼24.91, P < 0.001; time x group interaction F10,100 ¼ 25.54, P < 0.001). Pretreatment with JMV2959 3 and 6 mg/kg significantly and practically coincidently augmented the challenge morphine-induced HVA increase to 144% and 146% respectively of baseline 40 min post-morphine injection (JMV2959 3 mg/kg þ challenge morphine 5 mg/kg vs. saline þ challenge morphine 5 mg/kg: effect of group n.s.; effect of time F10,100 ¼ 73.15, P < 0.001; time x group interaction F10,100 ¼ 5.73, P < 0.001; JMV2959 6 mg/kg þ challenge morphine 5 mg/kg vs. saline þ challenge morphine 5 mg/kg: ef- fect of group n.s.; effect of time F10,100 ¼ 96.51, P < 0.001; time x group interaction F10,100 ¼ 8.21, P < 0.001). The single challenge dose 3 mg/kg JMV2959 slightly increased the accumbens HVA during 80 and 100 min intervals after challenge saline at the edge of significance (104% of baseline mean) (JMV2959 3 mg/kg þ challenge saline vs. saline þ challenge saline: effect of group n.s.; effect of time F10,100¼2.97, P < 0.05; time x group interaction F10,100 ¼ 2.68, P < 0.05). The higher dose 6 mg/kg JMV2959 also slightly, temporarily significantly increased accumbens HVA concentrations, with maximum of 111% of baseline level (JMV2959 6 mg/kg þ challenge saline vs. saline þ challenge saline: effect of group F1,10 ¼ 20.52, P < 0.001; effect of time F10,100 ¼ 11.88, P < 0.001; time x group interaction F10,100 ¼ 20.50, P < 0.001). Application of challenge saline did not change HVA concentrations.

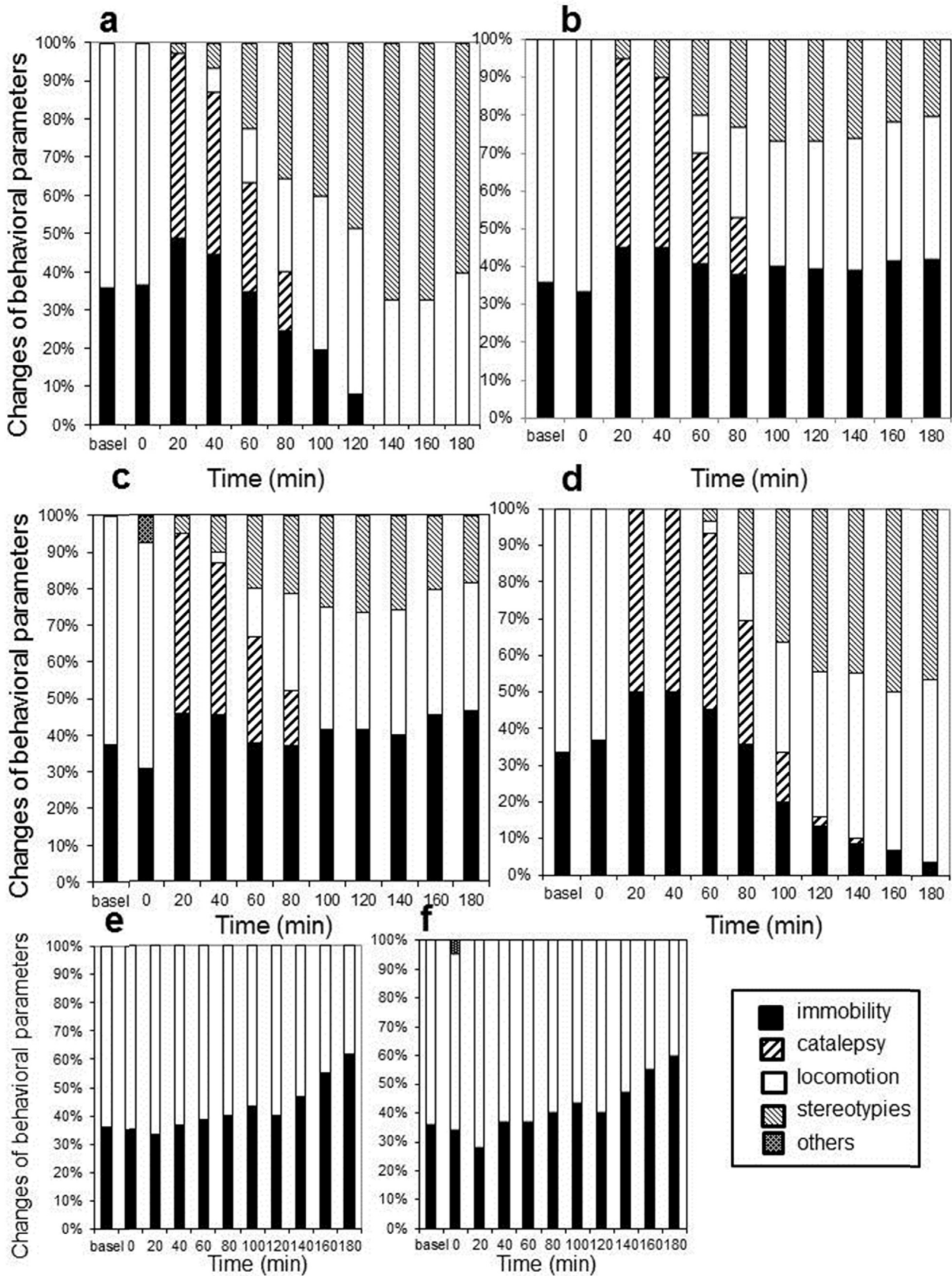

Fig. 4. The effects of GHS-R1A antagonist on challenge morphine-induced behavioral changes in five different observed parameters: immobility (black), catalepsy (thick diagonal strips), locomotion (white), stereotyped activity (thin diagonal strips), and others/stretching-like movements (grid pattern) are illustrated as a percentage of total behavior during each 20 min interval (total behaviors ¼ 100%). The means from n ¼ 6 rats are presented. JMV2959 was applied i.p. 20 min before morphine/saline. The behavioral changes are shown with respect to baseline conditions (mean of 60 min of baseline period) (basel ¼ control), the time interval after pretreatment with JMV2959/saline (0), is followed by 20e180 min

3.2. Behavioral assay

The effects of GHS-R1A antagonist on the challenge morphine-induced behavioral sensitization are illustrated in Fig. 4aef. Observed biphasic (inhibition-stimulation) morphine-induced changes in rats during the above described microdialysis experiments are shown with respect to baseline conditions (mean of 60 min ¼ three 20-min intervals of baseline periodebaseline control values); 0 min illustrates the time interval after pretreatment with JMV2959 3 or 6 mg/kg or saline, followed by 20e180 min of the morphine or saline effect (in accordance with (Fiserova et al., 1999; Sustkova-Fiserova et al., 2014). As expected, in comparison to acute morphine effects (Fig. 4d), clear signs of behavioral sensitization were observed when 5 mg/kg challenge morphine was administered on the 12th day of abstinence following longer-term morphine treatment (Fig. 4a). We observed an increase on the stereotypical sniffing, gnawing and licking, increased rearing and walking, shortened catalepsy. However, this study has been mainly focused on monitoring of JMV2959 pretreatment effects. These results are in accordance with our previously presented article (behavioral scores) (Sustkova-Fiserova et al., 2016). The effects of 3 and 6 mg/kg JMV2959 pretreatment (shown in Fig. 4b and c) seem practically identical: the challenge morphine-induced stereotypical behaviors and increased locomotion were significantly reduced and immobility increased 80e180 min after morphine. The two-way ANOVA for repeated measures (RM) followed by Bonferroni's test revealed the following significances - for the lower JMV2959 dose: JMV2959 3 mg/kg þ challenge morphine 5 mg/kg vs. saline þ challenge morphine 5 mg/kg: *immobility*: effect of group F1,10 ¼ 53.95, P < 0.001; effect of time F10,100 ¼ 29.17, P < 0.001; time × group interaction F10,100 ¼ 24.42 P < 0.001 at intervals 80e180 min after morphine; *catalepsy*: effect of group n.s.; effect of time F10,100 ¼ 363.40, P < 0.001; time × group interaction n.s.; *locomotion:* effect of group F1,10 ¼ 14.10, P < 0.01; effect of time F10,100 ¼ 243.36, P < 0.001; time × group interaction F10,100 ¼ 6.10 P < 0.001 at intervals 100e180 min after morphine; *stereotypies:* effect of group F1,10 ¼ 58.78, P < 0.001; effect of time F10,100 ¼ 152.22, P < 0.001; time × group interaction F10,100 ¼ 22.96 P < 0.001 at intervals 80e180 min after morphine. For the higher dose: JMV2959 6 mg/kg þ challenge morphine 5 mg/kg vs. saline þ challenge morphine 5 mg/kg: *immobility*: effect of group F1,10 ¼ 54.68, P < 0.001; effect of time F10,100 ¼ 27.44, P < 0.001; time × group interaction F10,100 ¼ 24.07 P < 0.001 at intervals 80e180 min after morphine; *catalepsy*: effect of group n.s.; effect of time F10,100 ¼ 327.81, P < 0.001; time × group interaction n.s.; *locomotion:* effect of group F1,10 ¼ 12.25, P < 0.01; effect of time F10,100 ¼ 229.11, P < 0.001; time × group interaction F10,100 ¼ 5.46 P < 0.001 at intervals 100e160 min after morphine; *stereotypies:* effect of group F1,10 ¼ 58.88, P < 0.001; effect of time F10,100 ¼ 155.30, P < 0.001; time × group interaction F10,100 ¼ 22.90 P < 0.001 at intervals 80e180 min after morphine. The 3 mg/kg JMV2959 which was administered before challenge saline did not induce any behavioral changes in the rats, the observed behavior was identical with saline treated rats. The 6 mg/kg JMV2959 administered before challenge saline (Fig. 4f) produced temporary behavioral changes in less than 40% of treated rats and always during the first interval (0 min). Typical stretching-like movements were fully eliminated with touch or sound during the first interval and spontaneously disappeared before morphine, or saline was administered (20 min after 6 mg/kg JMV2959), which is fully in accordance with acute effects of JMV2959 in Wistar rats (Sustkova-Fiserova et al., 2014). Similarly, to saline þ challenge saline-treated rats (Fig. 4e), during the last two intervals of dialysis (160e180 min), we observed increased immobility in the 3 and 6 mg/kg JMV2959 þ challenge saline-treated rats: 6 mg/kg JMV2959 þ saline vs. saline þ saline: in all monitored behavioral categories n.s., except as mentioned above for *other symptoms/stretching-like movements*: effect of group F1,10 ¼ 5.00, P ¼ 0.049; effect of time F10,100 ¼ 5.00, P < 0.001; time × group interaction F10,100 ¼ 5.00 P < 0.001 only at interval "0" after JMV2959 administration.

3.3. Conditioned place preference

In our experimental design in Wistar rats, the observed morphine-induced CPP was clearly manifested and it was significantly and dose dependently attenuated by acute single injections of 3 as well as 6 mg/kg JMV2959 on the post-conditioning day: F2,41 ¼ 8.11, P ¼ 0.001 (see Fig. 5). The rats were placed into the CPP chamber set 20 min after the JMV2959 or saline was administered.

3.4. Open field (Ethovision)

The rat explorative locomotor activity in the "open field" Ethovision test that was monitored for 20 min, was not significantly influenced by 1, 3 and 6 mg/kg JMV2959 when administered intraperitoneally 25 min before (see the Table 1.). The two-way RM ANOVA followed by Bonferroni's test revealed no significant differences among saline and the 3 doses of JMV2959 in trend of two dependent variables between the first and second 10min intervals of open-field experiment within distance and velocity parameters: time × group interaction F3,32 ¼ 0.598, P ¼ 0.621. The mean levels of monitored behavioral parameters within total 20min were not significant among all groups: F3,32 ¼ 0.954, P ¼ 0.426; (one-way ANOVA). Analogously the central zone stay duration parameter was also not significantly influenced by the administered doses of JMV2959: time × group interaction F3,32 ¼ 1.284, P ¼ 0.297 (two-way ANOVA RM) and F3,32 ¼ 1.186, P ¼ 0.331; (one-way ANOVA).

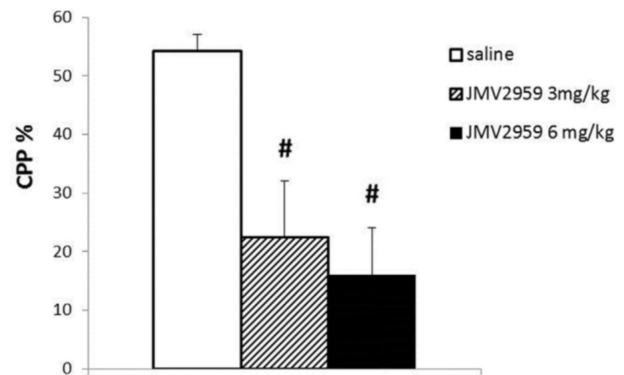

Fig. 5. Effects of GHS-R1A antagonist on the morphine-induced CPP in rats. After 8 days of conditioning with 10 mg/kg s.c. morphine, JMV2959 was administered in single dose 20 min before testing (N ¼ 15 in JMV groups, N ¼ 14 in the saline group; means ± SEM). Effects of JMV2959 pretreatments in comparison to the saline group are expressed as #P < 0.05.

of the morphine/saline effects (20e180 min). The results were obtained during dialysis in rats treated with the following: a saline þ challenge morphine 5 mg/kg, b JMV2959 3 mg/kg þ challenge morphine 5 mg/kg, c JMV2959 6 mg/kg þ challenge morphine 5 mg/kg, d saline þ morphine acute, e saline þ challenge saline, f JMV2959 6 mg/kg þ challenge saline. The observed behavior within the rat group JMV2959 3 mg/kg þ challenge saline was identical with the saline þ challenge saline group (not shown).

Table 1
Locomotor activity in “open field” within 20 min starting 25 min after intraperitoneal administration of JMV2959 (1, 3 and 6 mg/kg i.p.) did not significantly differ from the control, with saline treated group (N ¼ 9).

Distance

Interval	0-10min	10-20min	total 20min
saline	581.06 ± 86.07	292.28 ± 68.51	873.33 ± 142.34
JMV2959 1 mg/kg	672.41 ± 67.82	278.37 ± 44.17	950.78 ± 102.44
JMV2959 3 mg/kg	501.17 ± 55.27	221.15 ± 36.33	722.32 ± 80.05
JMV2959 6 mg/kg	437.40 ± 49.82	222.19 ± 31.07	720.00 ± 68.44

Velocity

Interval	0e10min	10e20min	total 20min
saline	0,97 ± 0.14	0.49 ± 0.11	0.73 ± 0.12
JMV2959 1 mg/kg	1.12 ± 0.11	0.46 ± 0.07	0.79 ± 0.09
JMV2959 3 mg/kg	0.84 ± 0.09	0.37 ± 0.06	0.60 ± 0.07
JMV2959 6 mg/kg	0.73 ± 0.08	0.37 ± 0.05	0.60 ± 0.06

Two-way ANOVA RM time × group interaction F3,32 ¼ 0.598, P ¼ 0.621 (n.s.)
One-way ANOVA within total 20min among all groups: F3,32 ¼ 0.954, P ¼ 0.426 (n.s.)

Central zone duration

Interval	0e10min	10e20min	total 20min
saline	16,24 ± 7.02	6.73 ± 3.90	22.97 ± 7.57
JMV2959 1 mg/kg	20.06 ± 6.90	10.06 ± 6.54	30.12 ± 10.27
JMV2959 3 mg/kg	4.19 ± 1.95	6.87 ± 4.06	11.05 ± 5.94
JMV2959 6 mg/kg	4.98 ± 2.35	8.26 ± 5.99	13.24 ± 24.43

Two-way ANOVA RM time × group interaction F3,32 ¼ 1.284, P ¼ 0.297 (n.s.)
One-way ANOVA within total 20min among all groups: F3,32 ¼ 1.186, P ¼ 0.331 (n.s.)

3.5. In vivo microdialysis in acute experiment

As illustrated in Fig. 6, in accordance with our previous experiments (Sustkova-Fiserova et al., 2014) the significant accumbens dopamine increase induced by acute morphine dose 10 mg/kg (saline þ acute morphine 10 mg/kg vs. saline þ saline: effect of group F1,10 ¼ 64.28, P < 0.001; effect of time F10,100 ¼ 53.67, P < 0.001; time × group interaction F10,100 ¼ 52.35, P < 0.001; two-way ANOVA RM Bonferroni) was reduced with pretreatment of JMV2959 3 mg/kg significantly within this new rat group (n ¼ 7) (JMV2959 3 mg/kg þ acute morphine 10 mg/kg vs. saline þ acute morphine 10 mg/kg: effect of group F1,11 ¼ 4.87, P < 0.001; effect of time F10,110 ¼ 188.06, P < 0.001; time × group interaction F10,110 ¼ 5.46, P < 0.001; two-way ANOVA RM Bonferroni). The maximal morphine induced dopamine increase 208% of baseline dropped to 179% when morphine was pretreated with 3 mg/kg JMV2959. Ghrelin 40 mg/kg co-administration with 3 mg/kg JMV2959 20 min before 10 mg/kg morphine has completely abolished the JMV2959 effects: (JMV2959 3 mg/kg together with ghrelin 40 mg/kg þ acute morphine 10 mg/kg vs. JMV2959 3 mg/kg þ acute morphine 10 mg/kg: effect of group F1,11 ¼ 126.48, P < 0.001; effect of time F10,110 ¼ 912.62, P < 0.001; time × group interaction F10,110 ¼ 4.76, P < 0.001; two-way ANOVA RM Bonferroni). Ghrelin co-administration even induced higher dopamine increase (with maximum 217% of baseline) than in the saline þ morphine 10 mg/kg rat group, although with low significant difference: JMV2959 3 mg/kg together with ghrelin þ morphine 10 mg/kg vs. saline þ morphine 10 mg/kg: effect of group F1,10 ¼ n.s., effect of time F10,100 ¼ 252.10, P < 0.001; time × group interaction F10,100 ¼ 2.27, P < 0.05. In accordance with the literature (Quarta et al., 2009), a single 40 mg/kg ghrelin i.p. induced significant accumbens dopamine increase with maximum of 191% 60 min after injection (saline þ ghrelin 40 mg/kg vs. saline þ saline: effect of group F1,10 ¼ 825.23, P < 0.001; effect of time F10,100 ¼ 123.36, P < 0.001; time × F10,100 ¼ 123.78, P < 0.001). Both combinations of JMV2959 with 10 mg/kg morphine induced significant accumbens dopamine increase (JMV 3 mg/kg þ acute morphine 10 mg/kg þ vs. saline þ saline: effect of group F1,11 ¼ 792.72, P < 0.001; effect of time F10,110 ¼ 127.21, P < 0.001; time × group interaction F10,100 ¼ 125.156, P < 0.001; JMV3mg/kg together with ghrelin 40 mg/kg þ morphine 10 mg/kg vs. saline þ saline: effect of group F1,10 ¼ 743.52, P < 0.001; effect of time F10,100 ¼ 348.97, P < 0.001; time × group interaction F10,100 ¼ 339.31, P < 0.001; two-way ANOVA RM Bonferroni). Single 3 mg/kg JMV2959 had no effect on accumbens dopamine.

4. Discussion and conclusion

The aim of this study was to ascertain if the GHS-R1A antagonism will reduce selected markers of opioid/morphine reinforcing properties, hence ghrelin antagonists might be considered as a potential new treatment strategy for opioid addiction.

In the first experiment, we have substantiated that challenge morphine-induced accumbens dopamine sensitization and behavioral sensitization in rats was significantly reduced by pretreatment with the systemic GHS-R1A antagonist substance JMV2959. Drug sensitization reflects a form of neuronal plasticity in which repeated drug administration leads to long-lasting increases in behavioral activation and dopamine overflow within the NAC and it is thought that these effects can lead to enhanced drug taking and addiction (Robinson and Berridge, 2003; Vezina, 2004, 2007). Drugs that increase accumbens dopamine overflow acutely but fail to produce sensitization of this effect when administered during a prolonged abstinence, are not associated with the subsequent enhancement of self-administration (Vezina, 2004). Mesolimbic dopaminergic system is critically involved in mediating both the incentive e motivational and sensitizing properties of opioids (Nakagawa et al., 2011; Robinson and Berridge, 1993a; Spanagel et al., 1993; Steketee and Kalivas, 2011; Vanderschuren and Kalivas, 2000). We have observed significant dopaminergic hypersensitivity to the challenge morphine dose administered on the 12th day of morphine abstinence. This is regarded as manifestation of dopaminergic sensitization mainly in the NAC core substructure, considering that protracted abstinence from non-associated repeated administration of opioids is characterized by an enhancement of drug-evoked accumbens dopamine release preferentially in the NAC core (Cadoni and Di Chiara, 1999; Di Chiara, 2002; Di Chiara et al., 2004; Spanagel et al., 1993). We have established for the first time that ghrelin antagonist significantly and dose-dependently reduced the morphine-evoked accumbens dopaminergic sensitization, which implies important participation of ghrelin at these processes and also potential treatment ability of ghrelin antagonism for opioid addiction, since, it is supposed that the accumbens dopaminergic hypersensitivity may underlie the reinstatement of compulsive drug-seeking behavior (Robinson and Berridge, 1993b; Spanagel et al., 1993; Vanderschuren and Kalivas, 2000). These results match our additional acute experiment, where involvement of ghrelin system in acute morphine induced accumbens dopamine increase was confirmed by co-administration of ghrelin. The significant reduction of morphine-induced accumbens dopamine release after pretreatment with 3 mg/kg JMV2959 was completely abolished by co-administration of ghrelin 40 mg/kg with 3 mg/kg JMV2959. So far, according to the literature, ghrelin antagonism of the mesolimbic dopamine was monitored in not sensitized experiments, where the accumbens dopamine release was induced by acute administration of alcohol (Jerlhag et al.,

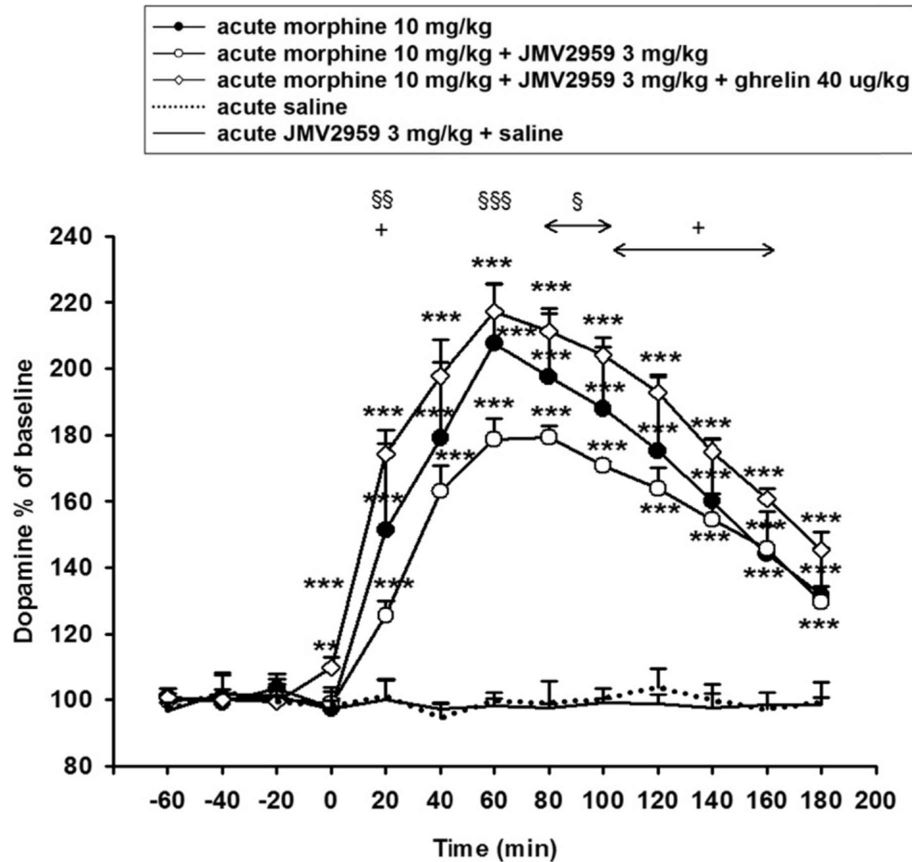

Fig. 6. Effect of ghrelin receptor GHS-R1A antagonist JMV2959 on acute 10 mg/kg morphine-induced dopamine in the rat NAC and its abolishment by ghrelin. 3 mg/kg JMV2959 together with saline or ghrelin 40 mg/kg was given i.p. 20 min before morphine/saline s.c. injection (n ¼ 6e7; means ± SEM). The effects are illustrated as follows: saline þ acute morphine (filled circle), 3 mg/kg JMV2959 þ acute morphine (open circle), 3 mg/kg JMV2959 together with 40 mg/kg ghrelin þ acute morphine (open diamonds), 3 mg/kg JMV2959 þ saline (continuous line), saline þ saline (dotting), effects of single ghrelin 40 mg/kg are not shown. Differences between treatments and baseline mean within a group are expressed as ***P < 0.001, **P < 0.01. Differences between groups saline þ acute morphine versus 3 mg/kg JMV2959 þ acute morphine or saline þ acute morphine versus 3 mg/kg JMV2959 together with 40 mg/kg ghrelin þ acute morphine effects are expressed as xxxP < 0.001, xxP < 0.01, xP < 0.05 or þ P < 0.05 respectively.

2009), cocaine, amphetamine (Jerlhag et al., 2010), nicotine (Jerlhag and Engel, 2011) and morphine (Engel et al., 2015; Sustkova-Fiserova et al., 2014) were also significantly reduced. In our previous experiments (Sustkova-Fiserova et al., 2014), the administration of JMV2959 3 as well as 6 mg/kg did not influence the accumbens dopamine, which is in accordance with the literature, where mainly the lower dose was used in rats (Jerlhag et al., 2012). Also JMV2959 3 mg/kg per se administered during abstinence from repeated saline did not influence the accumbens dopamine. The administration of the higher 6 mg/kg JMV2959 dose, which is considered rather high for rats, during saline abstinence moderately and transiently significantly decreased the accumbens dopamine. Yet, we observed practically identical effects of pretreatment with both JMV2959 3 and 6 mg/kg doses on the accubmens morphine-induced dopamine sensitization, the metabolite changes and behavioral sensitization and comparably significant effects on morphine-induced CPP in our Wistar rats. Thus, these JMV2959 changes are considered as morphine-induced GHS-R1A-participating blocking effects, which we have confirmed with a co-administration of ghrelin in our acute experiment.

Concerning the monitored metabolism of dopamine in the NAC, as expected, morphine administration increased significantly the extracellular accumbens dopamine turnover (Pothos et al., 1991; Westerink and Korf, 1976). The accumbens dopamine metabolites concentrations were increased when morphine was administered during abstinence in comparison to the acute effects, although the only significant difference was found in the HVA accumbens levels. Our results are roughly in accordance with the literature (Airio et al., 1994; Attila and Ahtee, 1984; Pothos et al., 1991). Our main interest was the effect of JMV2959 pretreatment on these morphine-induced changes. The significant JMV2959 reduction of challenge morphine-induced dopamine increase was associated with significant reduction of 3-MT (a product of catechol-Omethyltransferase (COMT)). On the other hand, concurrently we observed significant increase of DOPAC (a product of monoamine oxidase (MAO)) and significant increase of HVA, the final product of dopamine metabolism. JMV2959 6 mg/kg itself (per se) moderately increased levels of DOPAC and HVA, but did not influence that of 3-MT. Also the lower 3 mg/kg JMV2959 dose slightly and temporarily increased accumbens HVA. Thus, it seems that GHS-R1A antagonism might be associated with increased metabolism of dopamine by MAO. However, further investigation is required, as it has recently been described, that also ghrelin increases the turnover of dopamine in the NAC (Anderberg et al., 2016).

During our chronic microdialysis experiments, the behavioral changes were simultaneously monitored in the rats, in order to observe also the JMV2959 effects on the morphine-evoked behavioral sensitization. Behavioral sensitization can be used within certain limits as one of the tools for investigating the incentive motivation underlying drug-seeking behavior (Robinson and Berridge, 1993a; Steketee and Kalivas, 2011; Vanderschuren and Kalivas, 2000; Vanderschuren et al., 1999). Morphine induces

dose-dependent biphasic inhibitory-stimulatory effects and typical stereotyped behavior changes in rats (Fiserova et al., 1999; Koob and Volkow, 2010; Sustkova-Fiserova et al., 2014; Wise and Bozarth, 1987). In our study, within the four main monitored behavioral categories (stereotypies, locomotion, catalepsy, immobility), pretreatment with JMV2959 significantly reduced the challenge morphine-sensitized exhibition of stereotypies and locomotion percentage, together with the proportionally increased immobility percentage. These results match our previous studies, where involvement of ghrelin system in opioid induced stereotypies and increased locomotion was confirmed by co-administration of ghrelin in the acute experiment (Sustkova-Fiserova et al., 2016). Cross sensitization was described between ghrelin and cocaine in rats (Wellman et al., 2008). Pharmacological inactivation of GHS-R1A receptors (using JMV2959 or knockout animals) was already described to attenuate induction of behavioral sensitization to nicotine (Wellman et al., 2011), cocaine (Abizaid et al., 2011; Clifford et al., 2012) and ethanol (Bahi et al., 2013), thus our results indicate the ghrelin participation in the opioid-induced behavioral sensitization process, hence in opioid reinforcing processes.

Despite some limitations, CPP provides unique information about the rewarding effect of contextual cues associated with a drug stimulus (Bardo and Bevins, 2000) assessing the reinforcing effects of drugs, including opioids (Bals-Kubik et al., 1993; Shippenberg et al., 1993). Ghrelin co-administration increased cocaine-induced CPP in rats (Davis et al., 2007). Either genetic or pharmacological suppression of GHS-R1A, using ghrelin knock-out mice or JMV2959 pretreatment in mice, reduced the conditioned place preference induced by ethanol (Bahi et al., 2013; Jerlhag et al., 2009), nicotine (Jerlhag and Engel, 2011), cocaine (Abizaid et al., 2011; Jerlhag et al., 2010), amphetamine (Jerlhag et al., 2010) and morphine (Engel et al., 2015). In our study, administration of 3 and 6 mg/kg JMV2959 significantly and dose-dependently decreased the convincing biased morphine-induced CPP in rats, which strongly implicates ghrelin signaling in opioid reward processes and indicates that ghrelin antagonism significantly attenuated morphine reinforcing properties. The single 1, 3 and 6 mg/kg JMV2959 doses did not significantly influence the rat locomotor activity 25min after the JMV2959 administration.

Collectively our results encourage further investigation of ghrelin antagonism as a potential novel strategy for opioid addiction therapy.

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ORIGINAL INVESTIGATION

Ghrelin and endocannabinoids participation in morphine-induced effects in the rat nucleus accumbens

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Abstract

Rationale and objectives In addition to dopamine, endocannabinoids are thought to participate in neural reward mechanisms of opioids. Number of recent studies suggests crucial involvement of ghrelin in some addictive drugs effects. Our previous results showed that ghrelin participates in morphine-induced changes in the mesolimbic dopaminergic system associated with reward processing. The goal of the present study was to test whether the growth hormone secretagogue receptor (GHS-R1A) antagonist JMV2959 was able to influence morphine-induced effects on anandamide (N-arachidonoyleth a nolamine, AEA) a nd 2-arachidonoylglycerol (2-AG) in the nucleus accumbens shell (NACSh).

Methods We used in vivo microdialysis to determine changes in levels of AEA and 2-AG in the NACSh in rats following (i) an acute morphine dose (5, 10 mg/kg s.c.) with and without JMV2959 pretreatment (3, 6 mg/kg i.p.) or (ii) a morphine challenge dose (5 mg/kg s.c.) with and without JMV2959 (3, 6 mg/kg i.p.) pretreatment, administered during abstinence following repeated doses of morphine (5 days, 10–40 mg/kg). Co-administration of ghrelin (40 ug/kg i.p.) was used to verify the ghrelin mechanisms involvement.

Results Pretreatment with JMV2959 significantly and dose-dependently reversed morphine-induced anandamide increases in the NACSh in both the acute and longer-term models, resulting in a significant AEA decrease. JMV2959 significantly intensified acute morphine-induced decreases in accumbens 2-AG levels and attenuated morphine challenge-induced 2-AG decreases. JMV2959 pretreatment significantly reduced concurrent morphine challenge-induced behavioral sensitization. JMV2959 pretreatment effects were abolished by co-administration of ghrelin.

Conclusions Our results indicate significant involvement of ghrelin signaling in morphine-induced endocannabinoid changes in the NACSh.

Keywords Morphine · Ghrelin · Endocannabinoids · Anandamide · 2-Arachidonoylglycerol · Neural reward system · Nucleus accumbens shell · Acute · Challenge during abstinence · Stereotyped behavior · Microdialysis

Introduction

An orexigenic peptide ghrelin (Kojima et al. 1999), a natural ligand of the growth hormone secretagogue receptor (GHS-R1A), is released mainly from the stomach and, to a lesser degree, by the CNS (e.g., hypothalamus). An increasing number of recent studies suggests that ghrelin plays an important role in alcohol and stimulant abuse; however, available literature implicating ghrelin in opioid use disorders is limited and inconclusive (Maric et al. 2012; D'Cunha et al. 2013; Sustkova-Fiserova et al. 2014). The only opioid/heroin self-administration study (Maric et al. 2012) showed that ghrelin (i.c.v.) was able to increase heroin intake, yet pretreatment with the peptide GHS-R1A antagonist (i.c.v.) did not influence heroin self-administration. However, in our previous study (Sustkova-Fiserova et al. 2014), we demonstrated that pretreatment with the GHS-R1A antagonist JMV2959, a non-peptidic triazole substance (Moulin et al. 2007), significantly

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and dose-dependently reduced acute morphine-induced dopamine release in the nucleus accumbens shell (NACSh), the region critically involved in mediating drug reward (Di Chiara and Imperato 1988; Di Chiara 2002) and also morphine-induced behavioral stimulation, especially stereotypical behaviors. These results indicate a significant role for ghrelin signaling in the morphine/opiod-induced changes observed in the mesolimbic dopaminergic (DA) system, which are associated with neural reward processing.

It has been documented that the endogenous cannabinoid system also plays an important role in reinforcing processes of opioids. For example, CB1 antagonists such as SR141716A (Rimonabant) decreased the opioid rewarding effects in both intravenous self-administration (Navarro et al. 2001; Caille and Parsons 2003; De Vries et al. 2003; Solinas et al. 2003) and conditioned place preference procedures (Chaperon et al. 1998; Navarro et al. 2001; Singh et al. 2004). CB1 agonist THC increased reinforcing effects of the intravenously self-administered heroin (Solinas et al. 2005). Caille and Parsons (2006) suggested, that CB1 receptors modulate opioid reward through the ventral striatopallidal projection; they described that intravenous heroin self-administration was significantly reduced by intra-accumbens shell infusions of the CB1 antagonist SR141716A. However, there was no significant effect of systemic SR141716A pretreatment on morphine-induced increases in the NACSh dopamine efflux (also Caille and Parsons 2003; Tanda et al. 1997). These findings suggest that accumbens CB1 receptors significantly modulate opioid reinforcement through DA-independent mechanisms (Caille et al. 2007). Caille et al. (2007) also described that heroin self-administration significantly increased dialysate N-arachidonoylethanolamine (anandamide, AEA) levels and simultaneously induced moderate but significant decrease in dialysate 2-arachidonoyl glycerol (2-AG) levels in the NACSh. This is in accordance with Vigano et al. (2004) measurements of accumbens AEA and 2-AG contents after repeated morphine administration in rats. Thus, endocannabinoids involvement in the motivational properties of heroin/opioids was supported, and it has been suggested that possibly particularly opioid-induced anandamide increase in the NACSh participates in opioid reward (Caille et al. 2007).

Anandamide (AEA) (Devane et al. 1992) and 2-AG (Mechoulam et al. 1995; Sugiura et al. 1995) are the best-characterized endocannabinoids. AEA and 2-AG differ in many ways, e.g., they appear to be formed under different conditions in various brain structures and are uniquely affected by different stimuli, including pharmacological stimulation (Freund et al. 2003; Piomelli 2003; Fride 2005; Di Marzo and Petrosino 2007; Solinas et al. 2008). Endocannabinoids are formed Bon demand,^ and they act as retrograde messengers in the CNS (Di Marzo et al. 1994; Piomelli 2003; Piomelli et al. 2006). As it was already mentioned above, opioids trigger the release of AEA in the NAC but the importance of this release remains unclear given that compounds that increased brain concentrations of AEA and prolonged AEA's actions did not alter heroin self-administration in rats (Solinas et al. 2005). Even less is so far known about the role of 2-AG in reward processes.

To our knowledge, the interaction between ghrelin and CB1 receptors or endocannabinoids in the NAC has thus far not been studied. We have found only a few studies supporting important interactions between cannabinoid and ghrelin signaling pathways in the regulation of food intake by the brain/hypothalamus-gut axis (Konturek et al. 2004; Cani et al. 2004; Tucci et al. 2004; Kola et al. 2008).

During opioid sensitization and/or self-administration, the accumbens AEA was increased, and 2-AG was decreased (Vigano et al. 2004; Caille et al. 2007). The aim of our present study was to test whether the GHS-R1A antagonist JMV2959 was able to influence morphine-induced effects on anandamide and 2-AG in the NACSh, when morphine was administered in acute or in challenge doses during abstinence. To complete our neurochemical findings, which we consider as the main contribution of our study, simultaneously, the behavioral changes were monitored during the in vivo microdialysis.

Effects of JMV2959 pretreatment on acute morphine-induced behavioral changes were partly described in our previous study in rats (Sustkova-Fiserova et al. 2014), when behavioral stimulation and especially morphine-induced stereotypical behaviors were significantly reduced by the GHS-R1A antagonist. These results are in accordance with the literature, where pharmacological inactivation of GHS-R1A (using JMV2959 or knockout animals) has been noted to attenuate or eliminate behavioral stimulation induced by acute ethanol and psychostimulants (Jerlhag et al. 2010, 2011; Jerlhag and Engel 2011; Wellman et al. 2013) and to attenuate induction of behavioral sensitization to nicotine (Wellman et al. 2011) and cocaine (Abizaid et al. 2011). Several studies assessed cross-sensitization between opiates and cannabinoids (Pontieri et al. 2001a,b; Cadoni et al. 2001; Singh et al. 2005). Vigano et al. (2004) described that SR141617A attenuated the behavioral manifestation of morphine sensitization but not its development and suggested that changes in endocannabinoid signaling play a role in morphine behavioral sensitization. To our knowledge, the effects of ghrelin antagonism on opioid-induced sensitization have not yet been tested; thus, we wanted also to test the influence of JMV2959 pretreatment on the behavioral manifestation of morphine-induced sensitization.

Materials and methods

Animals

Male Wistar rats (Velaz, Anlab Czech Republic), approximately 8-week old, weighing 200–240 g were used. Animals were given free access to food and water, and were housed in

polycarbonate cages with constant humidity (50–60 %), at room temperature (22–24 °C), and on a 12/12-h light/dark cycle, for at least one week before the experiments, which were performed from 8 a.m. to 3 p.m. Groups of six rats were used for each treatment, with one exception of ten rats in the group from the longer-term experiments: saline+morphine 5 mg/kg. Procedures involving animals, along with their care, were conducted in compliance with international laws; protocols respected the guidelines of the European Union Council (86/609/EU) and followed animal care instructions set forth by the national committee for the Care and Use of Laboratory Animals. Experiments were approved by the Expert Committee for Protection of Experimental Animals at the Third Faculty of Medicine, Charles University in Prague and were performed in accordance with the Animal Protection Act of the Czech Republic (No. 246/1992 Sb).

Drugs and chemicals

All reagents were analytical grade. Morphine hydrochloride was purchased from Dr. Kulich Pharma (CR) and rat ghrelin from Essence Line (CR). JMV2959 (1,2,4-triazole derivate), which has been demonstrated to be a GHS-R1A antagonist (Moulin et al. 2007), was kindly provided by Anton Bespalov, AbbVie (Germany). The substances were dissolved in saline, and saline was used as a control; volumes of 0.1–0.2 ml/100 g of body weight were used for administration to the rats.

In rats, reliably rewarding doses of morphine (Mackey and Van der Kooy 1985; Mucha and Herz 1986) 5 or 10 mg/kg were administered subcutaneously (s.c.). The selected doses of JMV2959 (3 and 6 mg/kg) were determined based on our previous study (Sustkova-Fiserova et al. 2014) and the literature (Jerlhag et al. 2010; Clifford et al. 2012). The higher JMV2959 dose (6 mg/kg) produced temporary behavioral changes in less than 40 % of treated rats, which could be fully eliminated with sound or touch and spontaneously disappeared within 20 min after administration. JMV2959 was administered intraperitoneally (i.p.) always 20 min prior to morphine or saline injections. Ghrelin dose 40 μg/kg i.p. was chosen as a standard eating-stimulatory dose (Arnold et al. 2006; Quarta et al. 2009) confirmed in our pilot study in Wistar rats.

Schedule of experiments

The acute effects of morphine, in rats, after pretreatment with JMV2959 or saline were monitored using in vivo microdialysis of the nucleus accumbens shell (NACSh). To verify the involvement of ghrelin antagonism in the tested mechanisms, in one rat group, ghrelin was administered together with JMV2959 intraperitoneally separately in the opposite sites. Simultaneously with dialysis, the same animals were monitored for morphine-induced behavioral changes. Treatment groups in the acute experiment were as follows: saline+saline, saline+ morphine 5 mg/kg, saline+ morphine 10 mg/kg, JMV2959 6 mg/kg+ saline, JMV2959 6 mg/kg+morphine 5 mg/kg, JMV2959 6 mg/kg+morphine 10 mg/kg, JMV2959 3 mg/kg+morphine 10 mg/kg, and JMV2959 3 mg/kg together with ghrelin+morphine 10 mg/kg.

In the second, experiment morphine was administered once a day for 5 days in increasing doses (10, 20, 20, 40, and 40 mg/kg s.c.) followed by period of abstinence. On the 12th day of abstinence, a morphine challenge dose (5 mg/kg s.c.) was given after pretreatment with JMV2959 (3 or 6 mg/kg i.p.) or saline, and the appropriate changes in the NACSh were monitored, again using in vivo microdialysis combined with simultaneous observation to note any induced behavioral changes in the animals.

The dialysis samples were always collected at 20-min intervals for a total of 260 min. Dialysates were analyzed for the concentration of AEA and 2-AG using high-sensitivity liquid chromatography combined with mass spectrometry.

In vivo microdialysis: assay of endocannabinoids

Surgery

The technique is described in full detail in Sustkova-Fiserova et al. (2014). Forty-eight hours before microdialysis, under ketamine–xylazine anesthesia (ketamine 100 mg/kg i.p., Narketan 100 mg/ml Vetoquinol; xylazine 10 mg/kg i.p., Xylapan 20 mg/ml, Vetoquinol), a disposable dialysis guide cannula (MAB4 probes, Agnthos, Sweden) was implanted into the rat NACSh (A: +2.0 mm and L: ±1.2 mm from the bregma and V: 6.2 mm from the occipital bone) (Paxinos and Watson 2007), using a stereotaxic instrument (Stoelting Co). The guide was randomly alternated on the left and right side. After surgery, the rats were kept in individual cages. After completion of the microdialysis experiments, placement of the dialysis probe was verified histologically (Fig. 1). Animals with the probe outside the shell region were excluded from the experiment.

Microdialysis and chemical analysis assay

On the day of the microdialysis experiment, a probe (MAB4, 2 mm active cuprophane membrane, Agnthos) was inserted into the guide cannula, and artificial cerebrospinal fluid (Ringer's solution; 147 mM NaCl, 2.2 mM $CaCl_2$ and 4.0 mM KCl; adjusted to pH 7.0) was flushed through the probe at a constant rate of 2.0 μl/min (Univentor 864 Syringe Pump, Agnthos). After minimum 40 min of habituation to the microdialysis setup (dialysate was discarded), 20-μl samples were collected at 20-min intervals in small polyethylene test tubes. The other 20-μl part of each interval dialysate was used for detection of other neurotransmitters. Some of these results have been published (Sustkova-Fiserova et al. 2014) or are in progression. After three consecutive baseline samples, rats were injected with saline or JMV2959 (i.p.), which was

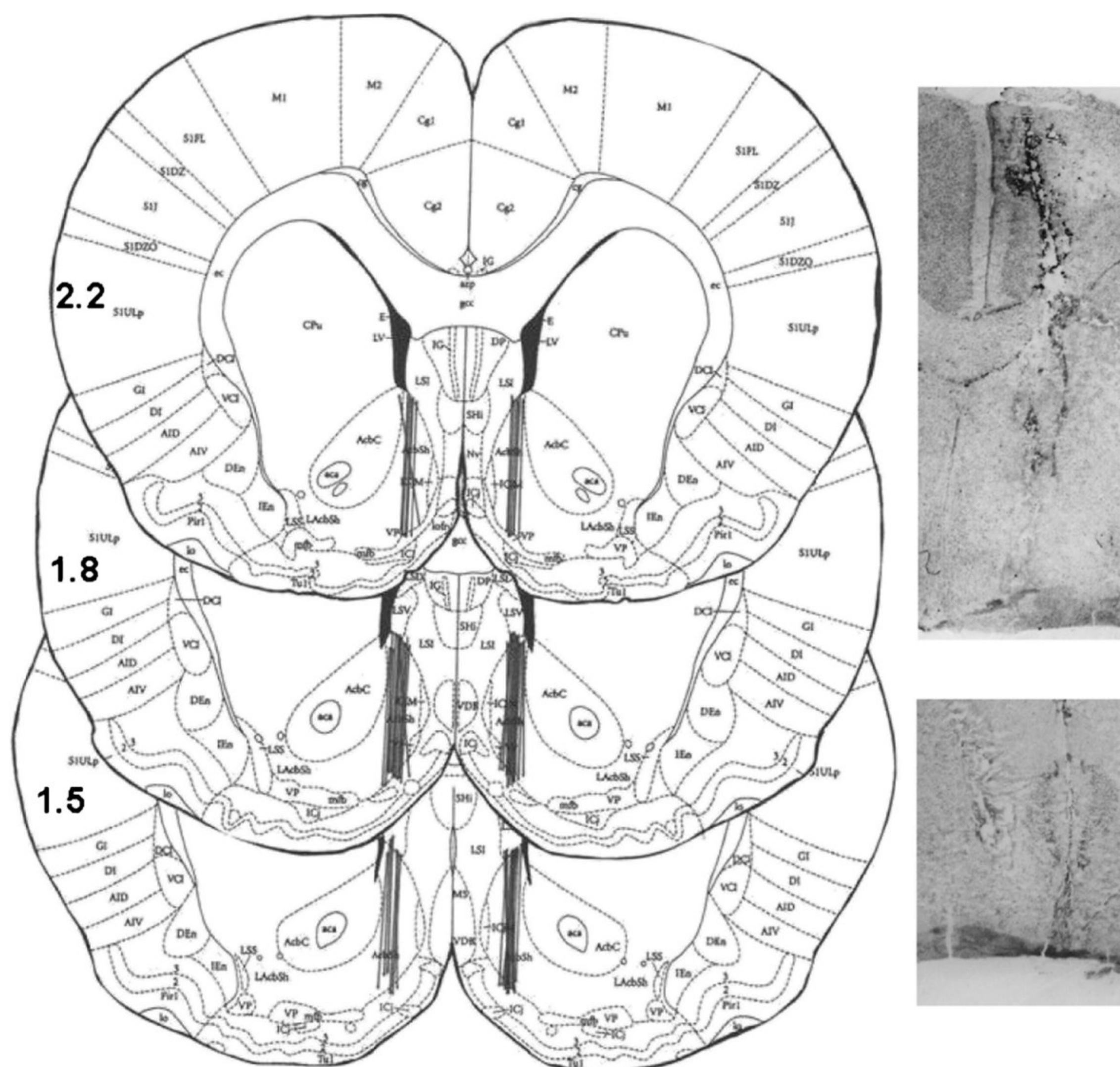

Fig. 1 Location of dialysis probes within the nucleus accumbens shell (NACSh). Schematic locations of probe tips in rats which were included in analyses of accumbens neurotransmitter concentrations (the *solid lines* indicate the dialyzing portions) as described in the atlas of Paxinos and Watson (2007). On the left, for each section, the distance from the bregma (in mm) is indicated. Representative photomicrographs on the right side show dialysis probe placements in the NACSh; only most ventral cannula track contains active dialysis membrane

followed (20 min later) by morphine or saline (s.c.) injection (in separate experiments). Samples were collected for 3 h following injection of morphine or saline. Immediately following collection, the samples were frozen at −80 °C. The amount of AEA and 2-AG in the dialysate was quantified using HPLC−MS. The method for determination of the endocannabinoids in the dialysate consisted of lyophilization to concentrate the substances from the dialysates, and detection using liquid chromatography combined with electrospray ionization tandem mass spectrometry (LC−ESI−MS/MS). Thus, the frozen microdialysate sample (−80 °C) with artificially added internal standard (IS), i.e., deuterium-labeled standards (2-AG-d_8 and AEA-d_8, 100 pg of each one) was inserted into a freeze dryer (Labconco FreeZone, USA) for 2 h. The freeze dryer condenser coil was cooled to −47 °C, and the pressure in the device was stabilized at 9 kPa. The lyophilization residue was dissolved while vortexing in acetonitrile (50 μl), causing precipitation of residual salts, peptides, etc. Suspensions of precipitated salts were centrifuged (2 min; 700×g), and the supernatant was immediately analyzed using a LC−ESI−MS/MS. The LC−MS system consisted of an Accela 1250 chromatograph (Thermo Scientific, USA), an Accela autosampler (Thermo Scientific), and a TSQ Vantage mass spectrometer (Thermo Scientific). The analytes were separated on a Gemini 5 μm NX-C18 110 Å, LC Column (150×2 mm) using a mobile phase (solvent A: aqueous solution of ammonium acetate (0.1-M solution, pH 5.4); solvent B: acetonitrile) in isocratic elution (ratio of mobile phase 70 % of solution A and 30 % of solution B) at a flow rate of 150 μl/min. 2-AG was eluted with a retention time of 5.2 min and AEA with a 4.3 min retention time. The column temperature was maintained at 30 °C. The injection volume was 5 μl. The mass spectrometer equipped

with an electrospray ionization source was used for detection of cannabinoids and their deuterium-labeled internal standard (2-AG-d_8 and AEA-d_8) in positive ionization mode (ESI^+). The selective reaction monitoring (SRM) mode was used. The scan monitoring reactions (precursor ion→fragment ion) used for the analyses and their collision-induced dissociation (CID) energy were as follows: m/z=379.3→m/z=287.3 (CID=13.5 eV) for 2-AG, 387.3→295.3 (13.5 eV) for 2-AG-d_8, 347.6 → 287.3 (15.5 eV) for AEA, and 356.6→ 295.3 (15.5 eV) for AEA-d_8. The conditions on the mass spectrometer were optimized and were as follows: spray voltage 3000 V, temperature of ion transfer tube 300 °C, temperature of H-ESI vaporizer 300 °C, pressure of sheath gas (nitrogen) 35 psi, flow of auxiliary gas (nitrogen) 10 arbitrary units, S-Lens−60 V. The data were acquired and processed using Xcalibur 2.1.0 software (Thermo Scientific).

The in vitro recovery of anandamide (AEA) and 2-AG, always through three dialysis probes (MAB4, 2 mm, Agnthos), was measured in solutions of four different concentrations (0.5, 1.5, 5.0, and 10.0 ng/ml) of the endocannabinoids either each separately or both in one mixture, dissolved in the Ringer's solution. The total average recovery (mean of all four solutions) of AEA was 51.05±4.05 % (alone) and 50.78±3.68 % (in the mixture), respectively. The total average recovery of 2-AG was 53.34±5.46 % (alone) and 53.06±5.58 % (in the mixture), respectively. However, our in vivo detected extracellular concentrations in the NACSh oscillated around 0.9–3.0 ng/ml of anandamide and 0.1–0.7 ng/ml of 2-AG. The limit of quantification (LOQ) for AEA was 240 pg/ml, and the LOQ for 2-AG was 280 pg/ml. The in vitro recovery procedure was performed in accordance with Fiserova et al. (1999).

Behavioral assay

Similarly to our previous studies (Fiserova et al. 1999; Sustkova-Fiserova et al. 2014), behavior was studied simultaneously, in the same animals, while microdialysis measurements were being taken. The following behavioral categories were distinguished: immobility (sedation, eyes closed, akinesia, and reduced responsiveness to environmental cues), catalepsy (frozen postures, exophthalmos, and trunk rigidity), locomotion (non-stereotyped activity, sniffing, grooming, rearing, and walking), and stereotypical behaviors (confined gnawing, licking, and stereotypical sniffing) (also in accordance with Rasmussen et al. 1990; Acquas and Di Chiara 1992; Rada et al. 1996). Behavioral categories were scored every 20 min (at each microdialysis interval) by an observer who was blinded to the treatment. The intensity or incidence of any changes in behavioral parameters associated with each 20-min interval (occurrences of the parameters during the whole 20-min interval) was assessed using predefined anchor points on a 4-point scale: 0=absent/no incidents, 1=mild/1–5 incidents, 2=moderate/medium/6–10 incidents, and 3=marked/maximum/more than 11 incidents. Behavioral changes were monitored during the entire dialysis period: 60-min baseline, 20-min pretreatment and 3-h following morphine or saline injection.

Statistical analysis

Raw data for endocannabinoids, expressed as ng/ml/sample, not corrected for probe recovery, were transformed into a percentage of baseline levels (mean of three values prior to pretreatment). Changes in behavioral parameters, during 20-min intervals, were also analyzed. Time course neurochemical and behavioral data were statistically analyzed using SigmaStat 3.5, Systat Software, Inc., USA. For statistical differences between the appropriate treatment groups (JMV2959 + morphine), (saline+morphine), and (saline+saline) in acute and long-term experiments relative to time-related changes in the course of the in vivo microdialysis study, a two-way analysis of variance for repeated measures (ANOVA RM analysis) followed by Bonferroni corrected linear contrasts test was used. In this ANOVA, the group of rats was entered as the between-group factor and the time-points as repeated within-subject measures, to compare all treatments to baseline mean; 20-min intervals over 200 min of post-treatment. The possible difference between baseline samples from acute and longer-term experiments was evaluated using the t-test. All statistical tests were evaluated as two-sided at a significance level of 0.05 (P values of <0.05, <0.01 and <0.001 defined statistical significance). Results are presented as the mean±SEM.

Results

The effects of GHS-R1A antagonist on morphine-induced accumbens anandamide (AEA) extracellular concentration increase

Morphine administered in acute doses

The influence of ghrelin antagonism on acute morphine-induced extracellular AEA increase in the NACSh is illustrated in Fig. 2a,b. AEA baseline levels did not differ significantly between animals, including the acute and longer-term experiments (Fig. 2d). As expected, acute systemic morphine administration induced a statistically significant and dose-dependent increase of AEA in the NACSh. The two-way ANOVA for repeated measures (RM) followed by Bonferroni's test revealed a significant group effect: saline+morphine 5 mg/kg vs. saline+ saline group ($F_{1,10}$= 17.43, P= 0.002) and time ($F_{10,100}$=7.58, P<0.001); time course of AEA changes in the NACSh after saline/morphine 5 mg/kg administration differed significantly between the two groups of animals (time x group interaction, $F_{10,100}$=3.39, P< 0.001). The 5 mg/kg

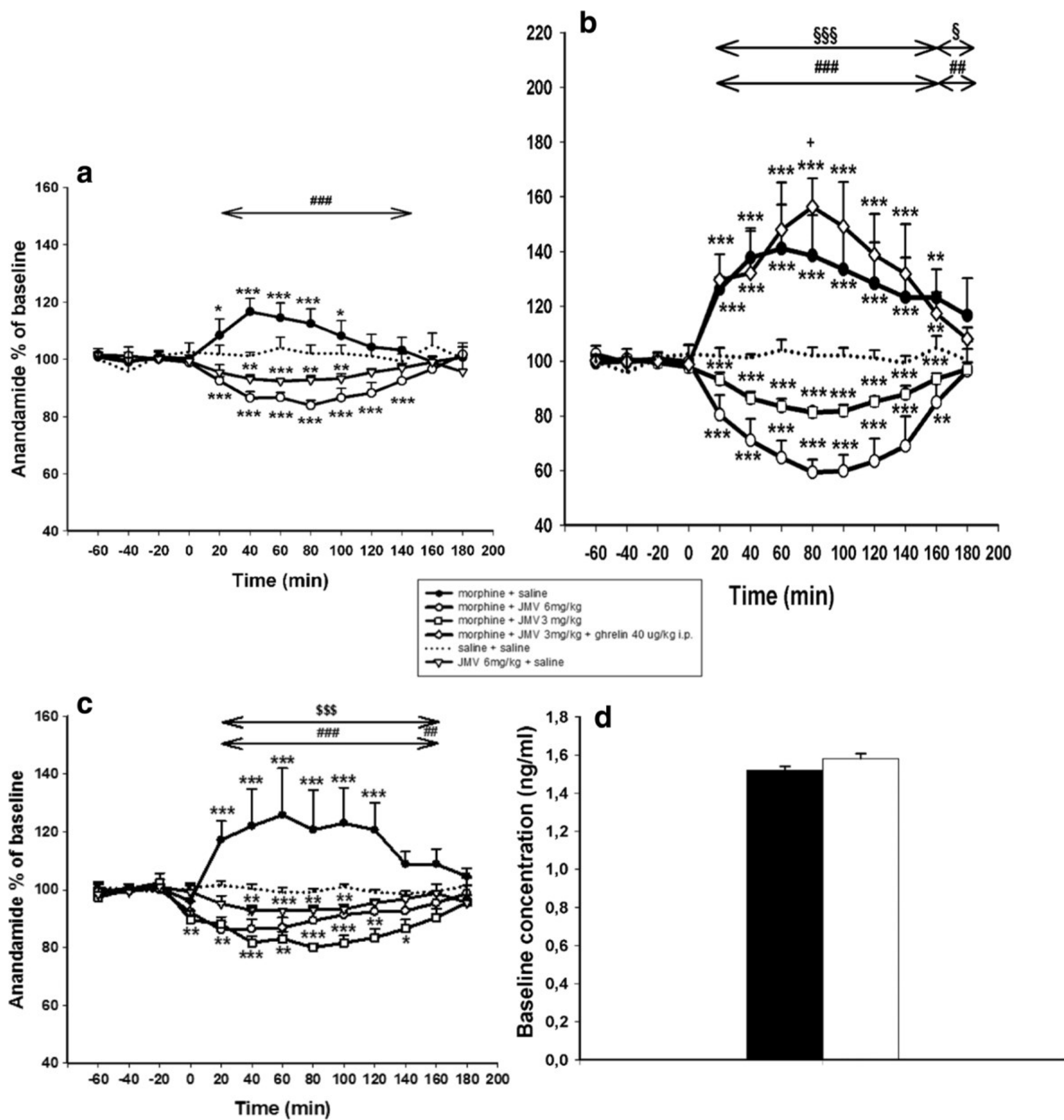

Fig. 2 Effects of ghrelin receptor GHS-R1A antagonist JMV2959 on morphine-induced AEA release in the rat NACSh. JMV2959 was always given i.p. 20 min before morphine/saline s.c. injection (n=6 for all groups with an exception of ten rats in the saline+challenge morphine group; means±SEM). The effects are illustrated as follows: (a) saline+ acute 5 mg/kg morphine (*filled circle*), 6 mg/kg JMV2959 + acute 5 mg/kg morphine (*open circle*), 6 mg/kg JMV2959+acute saline (*open triangle*), saline+ acute saline (dotting); (b) saline+ acute 10 mg/kg morphine (*filled circle*), 6 mg/kg JMV2959+acute 10 mg/kg morphine (*open circle*), 3 mg/kg JMV2959+ acute 10 mg/kg morphine (*open square*), 3 mg/kg JMV2959 together with ghrelin 40 μg/kg+ acute 10 mg/kg (*open diamond*), saline+acute saline (dotting); (c) saline+ challenge 5 mg/kg morphine (*filled circle*), 6 mg/kg JMV2959 + challenge 5 mg/kg morphine (*open circle*), 3 mg/kg JMV2959 + challenge 10 mg/kg morphine (*open square*), 6 mg/kg JMV2959 + challenge saline (*open triangle*), saline+saline challenge (dotting); (d) the differences between anandamide baselines in acute (*black bar*) and chronic (*white bar*) experiments (at P < 0.05, the difference was not significant). Differences between treatments and baseline mean are expressed as ***P<0.001, **P<0.01, *P<0.05. Differences between morphine and 6 mg/kg JMV2959+morphine or morphine and 3 mg/kg JMV2959+morphine or morphine and 3 mg/kg JMV2959 with ghrelin+ morphine effects are expressed as ###P<0.001, ##P<0.01, # P<0.05 or §§§P<0.001, §P<0.05 or+P<0.05, respectively

morphine-induced AEA increase in comparison to baseline mean within the rat group was also significant (P< 0.001), with the maximum effect of 117 % of baseline level. The larger morphine dose (10 mg/kg s.c.) induced a stronger accumbens AEA increase compared to the control/saline group: saline+morphine 10 mg/kg vs. saline+saline: effect of group $F_{1,10}$ = 28.86, P < 0.001; effect of time $F_{10,100}$ = 14.09, P<0.001; time x group interaction $F_{10,100}$=10.79, P<0.001 as well as to the baseline mean within the group, with maximum effect of 142 % of baseline level.

Pretreatment with the GHS-R1A antagonist JMV2959 reversed the morphine-induced accumbens AEA increase and

produced a significant and dose-dependent decrease. The 6 mg/kg JMV2959 pretreatment turned the 5 mg/kg morphine-induced AEA increase into a decrease with the maximum drop of 84 % of baseline level. Thus, the JMV2959 pretreatment effect was highly statistically significant: JMV2959 6 mg/kg+ morphine 5 mg/kg vs. saline+morphine 5 mg/kg: effect of group $F_{1,10} = 130.11$, $P < 0.001$; effect of time $F_{10,100} = 14.09$, $P<0.001$; time *x* group interaction $F_{10,100}=10.79$, $P<0.001$. Additionally, changes in comparison to baseline within the JMV2959 pretreatment group were significant ($P<0.001$). Three and 6 mg/kg JMV2959 pretreatment reversed dose-dependently and highly significantly as well as the 10 mg/kg morphine-induced AEA increase. For the lower JMV2959 dose: JMV2959 3 mg/kg+morphine 10 mg/kg vs. saline+morphine 10 mg/kg: effect of group $F_{1,10}=71.78$, $P<0.001$; effect of time $F_{10,100}=5.80$, $P<0.001$; time *x* group interaction $F_{10,100}= 32.55$, $P<0.001$. For the higher dose: JMV2959 6 mg/kg+morphine 10 mg/kg vs. saline+morphine 10 mg/kg: effect of group $F_{1,10} = 102.97$, $P < 0.001$; effect of time $F_{10,100} =2.36$, $P<0.001$; time *x* group interaction $F_{10,100}=43.13$, $P<0.001$. The AEA extracellular levels dropped in the JMV2959 pretreated groups dose-dependently to maximum of 81 % (3 mg/kg JMV2959 pretreatment) and 59 % (6 mg/kg JMV2959 pretreatment) of baseline level.

Ghrelin 40 μg/kg co-administration with 3 mg/kg JMV2959 20 min before 10 mg/kg morphine has completely abolished the JMV2959 effects: JMV2959 3 mg/kg together with ghrelin+ morphine 10 mg/kg vs. JMV2959 3 mg/kg+ morphine 10 mg/kg: effect of group $F_{1,10}=77.67$, $P<0.001$; effect of time $F_{10,100}=13.45$, $P<0.001$; time *x* group interaction $F_{10,100}=52.56$, $P<0.001$. Ghrelin co-administration even induced higher AEA increase than in the saline+morphine 10 mg/kg rat group, with significant difference 80 min after morphine injection: JMV2959 3 mg/kg together with ghrelin+ morphine 10 mg/kg vs. saline+morphine 10 mg/kg: effect of group $F_{1,10}$=n.s.; effect of time $F_{10,100}=44.42$, $P<0.001$; time *x* group interaction $F_{10,100}=2.66$, $P<0.05$. A single 40 μg/kg ghrelin i.p. induced significant AEA increase with maximum of 194 % 60 min after injection (not shown).

A single dose of JMV2959 6 mg/kg i.p. induced moderate, temporarily significant decrease of the accumbens AEA to maximum of 92 % of baseline level, 80-min post-injection ($P < 0.001$ compared to the baseline mean). JMV2959 6 mg/kg+saline vs. saline+saline: effect of group $F_{1,10}= 45.6$, $P< 0.001$; effect of time $F_{10,100}=$ n.s.; time *x* group interaction $F_{10,100}= 2.94$, $P = 0.003$. Application of acute saline had no effect on accumbens AEA.

Morphine administered during un-precipitated abstinence following repeated morphine treatment

The Fig. 2c illustrates the observed influence of ghrelin antagonism on changes in accumbens AEA induced by a 5 mg/kg morphine challenge applied on the 12th day of abstinence following 5 days of morphine treatment. In comparison with the acute 5 mg/kg morphine-induced effect, the same morphine dose applied as challenge dose during abstinence induced an AEA increase of higher intensity: saline+challenge morphine 5 mg/kg vs. saline+acute morphine 5 mg/kg: effect of group $F_{1,14}=6.61$, $P<0.05$; effect of time $F_{10,140}=16.64$, $P<0.001$; time *x* group interaction $F_{10,140}=2.43$, $P<0.05$. The challenge morphine AEA increase reached maximum of 126 % of baseline level and was significant ($P<0.001$), compared to baseline mean: saline+challenge morphine 5 mg/kg vs. saline+challenge saline: effect of group $F_{1,14}=27.37$, $P<0.001$; effect of time $F_{10,140}=8.24$, $P<0.001$; time *x* group interaction $F_{10,140}=8.80$, $P<0.001$ (N=10 rats in this group).

As with the acute situation, 3 and 6 mg/kg mg/kg i.p. JMV2959 pretreatment turned the challenge morphine-induced AEA increase into a decrease with the maximum drop of 82 % of baseline level in case of 3 mg/kg and 86 % in 6 mg/kg JMV2959 pretreatment ($P<0.001$). For the lower JMV2959 dose: JMV2959 3 mg/kg+challenge morphine 5 mg/kg vs. saline+challenge morphine 5 mg/kg: effect of group $F_{1,14}= 83.39$, $P<0.001$; effect of time $F_{10,140}=2.49$, $P<0.01$; time *x* group interaction $F_{10,140}=16.68$, $P<0.001$. For the higher JMV2959 dose: JMV2959 6 mg/kg+ challenge morphine 5 mg/kg vs. saline+challenge morphine 5 mg/kg: effect of group $F_{1,14}=68.02$, $P<0.001$; effect of time $F_{10,140}=4.21$, $P<0.001$; time *x* group interaction $F_{10,140}=15.10$, $P<0.001$.

A single dose of JMV2959 6 mg/kg i.p. during the morphine abstinence similarly to the acute situation temporarily significantly decreased the accumbens AEA to maximum of 93 % of baseline level, ($P<0.001$) compared to the baseline mean. JMV2959 6 mg/kg+challenge saline vs. saline+challenge saline: effect of group $F_{1,10}=63.62$, $P<0.001$; effect of time $F_{10,100}=4.35$, $P<0.001$; time *x* group interaction $F_{10,100}=4.77$, $P<0.001$. Application of challenge saline had no effect on accumbens AEA.

The effects of GHS-R1A antagonist on morphine-induced accumbens 2-arachidonoylglycerol (2-AG) extracellular concentration decrease

Morphine administered in acute doses

The influence of ghrelin antagonism on acute morphine-induced extracellular 2-AG decrease in the NACSh is illustrated in Fig. 3a,b. 2-AG baseline levels did not differ significantly between animals in the acute experiment. Acute morphine administration induced a statistically significant decrease of 2-AG in the NACSh. The two-way ANOVA for repeated measures followed by the Bonferroni test revealed a significant effect of group: saline+morphine 5 mg/kg vs. saline+saline group ($F_{1,10}=15.01$, $P=0.003$) and time ($F_{10,100}=4.90$, $P<0.001$); time course of 2-AG changes in the

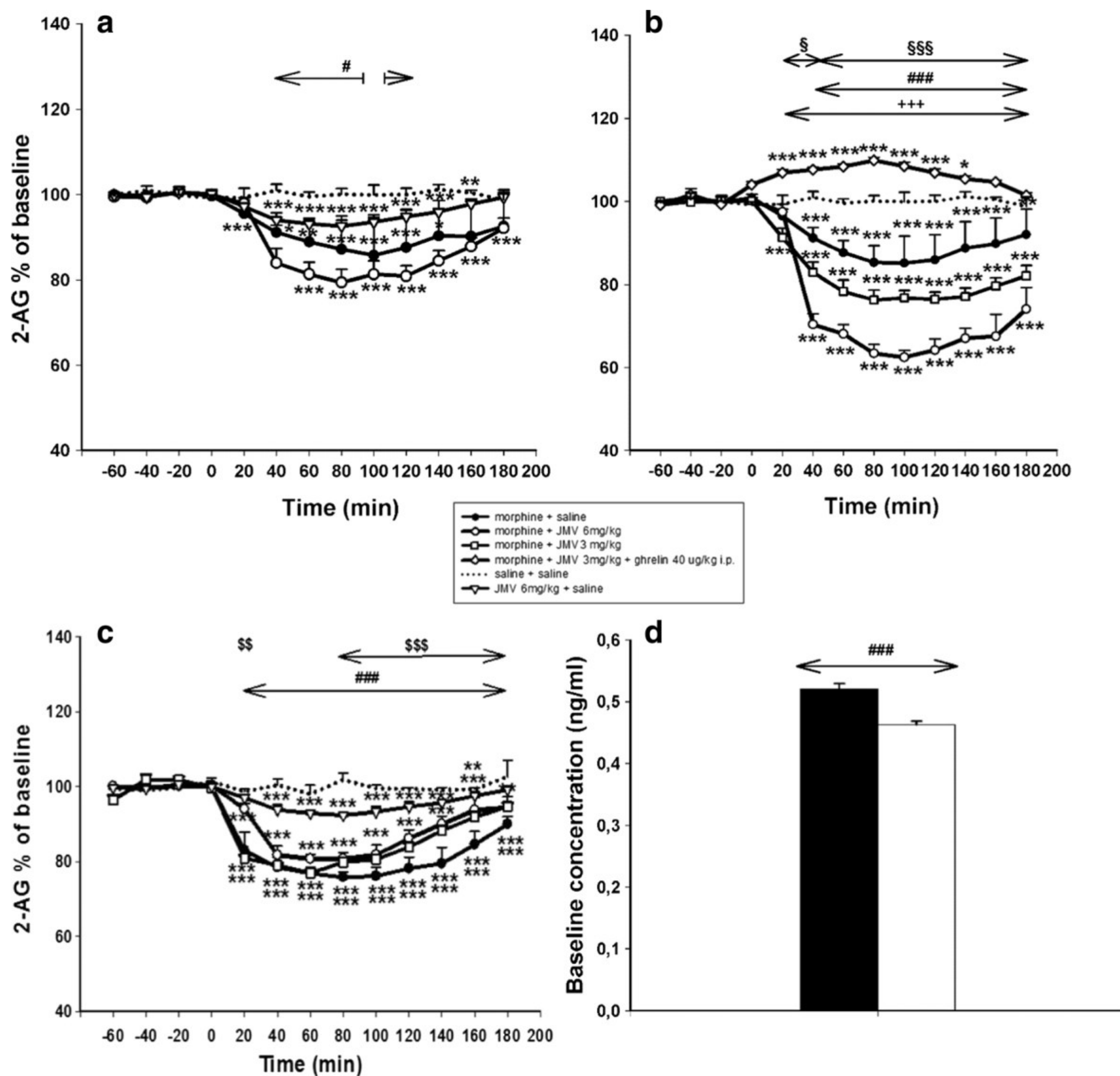

Fig. 3 Effects of ghrelin receptor GHS-R1A antagonist JMV2959 on morphine-induced 2-AG release in the rat NACSh. JMV2959 was always given i.p. 20 min before morphine/saline s.c. injection ($n=6$ for all groups with an exception of ten rats in the saline+challenge morphine group; means±SEM). The effects are illustrated as follows: (a) saline+ acute 5 mg/kg morphine (*filled circle*), 6 mg/kg JMV2959 + acute 5 mg/kg morphine (*open circle*), 6 mg/kg JMV2959+acute saline (*open triangle*), saline+ acute saline (dotting); (b) saline+ acute 10 mg/kg morphine (*filled circle*), 6 mg/kg JMV2959+acute 10 mg/kg morphine (*open circle*), 3 mg/kg JMV2959+ acute 10 mg/kg morphine (*open square*), 3 mg/kg JMV2959 together with ghrelin 40 μg/kg+ acute 10 mg/kg morphine (*open diamond*), saline+acute saline (dotting); (c) saline+5 mg/kg morphine challenge (*filled circle*), 6 mg/kg JMV2959+ 5 mg/kg MO challenge (*open circle*), 3 mg/kg JMV2959+ challenge 10 mg/kg morphine (*open square*), 6 mg/kg JMV2959 + challenge saline (*open triangle*), saline+ saline challenge (*dotting*); (d) the differences between 2-AG baselines in acute (*black bar*) and chronic (*white bar*) experiments (difference expressed as ###$P < 0.001$). Differences between treatments and baseline mean are expressed as ***$P<0.001$, **$P<0.01$, *$P<0.05$. Differences between morphine and 6 mg/kg JMV2959+morphine or morphine and 3 mg/kg JMV2959+ morphine or morphine and 3 mg/kg JMV2959 with ghrelin+morphine effects are expressed as ###$P<0.001$ or §§§$P<0.001$ or +++$P<0.001$, respectively

NACSh after saline/morphine 5 mg/kg administration differed significantly between the two groups of animals (time *x* group interaction, $F_{10,100}=5.61$, $P<0.001$). The 5 mg/kg morphine-induced 2-AG decrease in comparison to baseline mean within the group was also significant ($P<0.001$) with the maximum effect of 86 % of baseline level. The larger morphine dose 10 mg/kg induced comparable accumbens 2-AG decrease compared to the control/saline group: saline+MO 10 mg/kg vs. saline+saline: effect of group $F_{1,10}=34.65$, $P<0.001$; effect of time $F_{10,100}=12.35$, $P<0.001$; time *x* group interaction $F_{10,100}=13.92$, $P<0.001$, as well as to baseline mean within the group; maximum effect of 85 % of baseline level.

Pretreatment with JMV2959 dose-dependently deepened the morphine-induced accumbens 2-AG decrease. Six mg/kg i.p. JMV2959 pretreatment slightly but significantly ($P<0.05$) decreased the 5 mg/kg morphine-induced 2-AG drop into maximum of 79 % of baseline level: JMV2959 6 mg/kg+ morphine 5 mg/kg vs. saline+morphine 5 mg/kg: effect of

group F1,10=n.s.; effect of time F10,100=34.88, P<0.001; time x group interaction F10,100=2.65, P=0.007. Three and 6 mg/kg JMV2959 pretreatment intensified dose-dependently the 10 mg/kg morphine-induced 2-AG decrease. For the lower JMV2959 dose: JMV2959 3 mg/kg+morphine 10 mg/kg vs. saline+ morphine 10 mg/kg: effect of group F1,10= 24.38, P< 0.001; effect of time F10,100= 80.62, P<0.001; time x group interaction F10,100= 5.29, P< 0.001. For the higher JMV2959 dose: JMV2959 6 mg/kg+morphine 10 mg/kg vs. saline+morphine 10 mg/kg: effect of group F1,10=104.57, P<0.001; effect of time F10,100=114.91, P<0.001; time x group interaction F10,100=25.89, P<0.001. The 2-AG extracellular levels dropped in the JMV2959 pretreated groups dose-dependently to a maximum of 76 % (3 mg/kg JMV2959) and 62 % (mg/kg JMV2959) of baseline level. Ghrelin 40 μg/kg co-administration with 3 mg/kg JMV2959 20 min before 10 mg/kg morphine has completely abolished the JMV2959 effects: JMV2959 3 mg/kg together with ghrelin+ morphine 10 mg/kg vs. JMV2959 3 mg/kg+ morphine 10 mg/kg: effect of group F1,10=1467.32, P<0.001; effect of time F10,100=94.49, P<0.001; time x group interaction F10,100=199.35, P<0.001. Ghrelin co-administration even induced moderate but significant accumbens 2-AG increase in comparison to baseline mean, with maximum of 110 % (P<0.001). The 2-AG accumbens levels differed significantly from the saline+ morphine 10 mg/kg rat group: JMV2959 3 mg/kg together with ghrelin+morphine 10 mg/kg vs. saline+ morphine 10 mg/kg: effect of group F1,10 = 99.62, P < 0.001; effect of time F10,100 = 8.01, P < 0.001; time x group interaction F10,100=29.89, P<0.001. A single 40 μg/kg ghrelin i.p. induced significant 2-AG increase with maximum of 143 % 60 min after injection (not shown).

A single dose of JMV2959 6 mg/kg i.p. slightly but significantly decreased the accumbens 2-AG to a maximum of 93 % of baseline level (P < 0.001 compared to baseline mean). JMV2959 6 mg/kg+saline vs. saline+saline: effect of group F1,10 = 57.74, P < 0.001; effect of time F10,100 = 12.79, P<0.001; time x group interaction F10,100=17.38, P<0.001. Application of saline had no effect on accumbens 2-AG.

Morphine administered during un-precipitated abstinence following repeated morphine treatment

Figure 3c illustrates the observed influence of ghrelin antagonism on the accumbens 2-AG induced by 5 mg/kg morphine challenge dose given on the 12th day of abstinence from repeated morphine treatments. The 5 mg/kg morphine challenge dose induced a significant 2-AG decrease, with maximum of 75 % of baseline level (P<0.001 compared to baseline mean; N=10 rats in this group). Saline+challenge morphine 5 mg/kg vs. saline+challenge saline: effect of group F1,14=1026.50, P<0.001; effect of time F10,140=58.12, P<0.001; time x group interaction F10,140=52.27, P<0.001. The morphine-induced 2-AG decrease in the longer-term experiment was significantly deeper than in acute experiment: saline+challenge morphine 5 mg/kg vs. saline+acute morphine: effect of group F1,14= 25.12, P<0.001; effect of time F10,140=51.09, P<0.001; time x group interaction F10,140=5.77, P<0.001.

Pretreatment with 3 and 6 mg/kg JMV2959 slightly but significantly reduced the 5 mg/kg morphine challenge-induced 2-AG decrease, with maximum of 77 and 81 % of baseline level (P<0.001). For lower JMV2959 dose: 3 mg/kg JMV2959+5 mg/kg morphine challenge vs. saline+5 mg/kg morphine challenge: effect of group F1,14=28.13, P<0.001; effect of time F10,140=186.67, P<0.001; time x group interaction F10,140=7.80, P<0.001. For higher JMV2959 dose: 6 mg/kg JMV2959+5 mg/kg morphine challenge vs. saline+ 5 mg/kg morphine challenge: effect of group F1,14=78.50, P<0.001; effect of time F10,140=158.27, P<0.001; time x group interaction F10,140=6.07, P<0.001.

A single dose of JMV2959 6 mg/kg i.p. during the morphine abstinence similarly to the acute situation moderately but significantly decreased the accumbens 2-AG to maximum of 92 % of baseline level (P<0.001). JMV2959 6 mg/kg+challenge saline vs. saline+ challenge saline: effect of group F1,10= 166.72, P<0.001; effect of time F10,100=20.69, P<0.001; time x group interaction F10,100=15.75, P<0.001. Application of the saline challenge had no effect on accumbens 2-AG.

The baseline 2-AG concentrations did not differ among the animals within the chronic experiment. However, the basal levels in the longer-term experiment were significantly lower compared with 2-AG basal levels in the acute experiment (Fig. 3d); baselines acute vs. baselines chronic: T=6500.0, DF=208, P<0.001, two sample t-test.

The effects of GHS-R1A antagonist on morphine-induced behavioral changes

Morphine administered in acute doses

The observed biphasic (inhibition-stimulation) morphine-induced changes in rats during selected microdialysis experiments are illustrated in Figs. 4, 5, and 6. The behavioral changes (scores 0–3) are shown with respect to baseline conditions (mean of 60 min=three 20-min intervals of baseline period—baseline control values); 0 min illustrates the time interval after pretreatment with JMV2959 3 mg/kg or 6 mg/kg or saline, followed by 20–180 min of the morphine or saline effect. Morphine applied in all doses induced significant and expected changes in comparison to saline (in accordance with Fiserova et al. 1999; Sustkova-Fiserova et al. 2014). However, our main focus was on monitoring JMV2959 pretreatment effects (which are also marked in the graphs).

The acute 5 mg/kg morphine-induced changes with the pretreatments for each of the four monitored behavioral categories are shown in Fig. 4a–d. The most remarkable

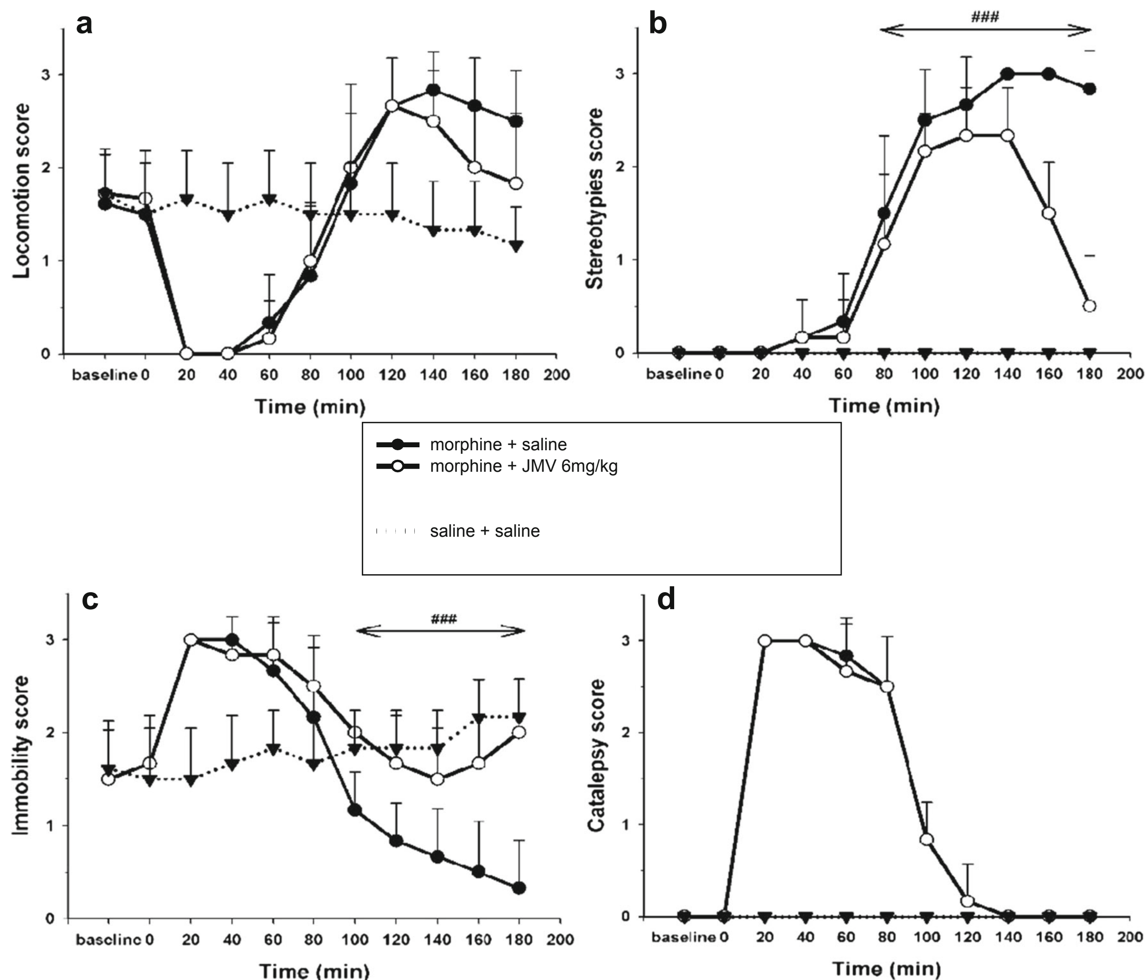

Fig. 4 a–d Effects of GHS-R1A antagonist JMV2959 (6 mg/kg) on acute 5 mg/kg morphine-induced behavioral changes in four observed categories are illustrated as means of behavioral scores (+/−SEM) separately: (a) locomotion, (b) stereotypies, (c) immobility, and (d) catalepsy. Behavioral changes are always shown with respect to baseline conditions (mean of 60 min of baseline period) (*baseline*), the time interval after pretreatment with JMV2959/saline (*0*) is followed by 20–180 min of morphine/saline effects (*20–180 min*). The behavioral effects are illustrated as follows: saline+ morphine (*filled circle*), JMV2959+morphine (*open circle*), saline+saline (*filled triangle with dotting*). Differences between morphine and JMV2959 + morphine effects are expressed as ###P < 0.001. (The saline+ acute JMV2959 6 mg/kg effects (not shown) were comparable with the saline+saline group, only within the 0–20-min interval after JMV2959 were writhing-like signs observed; however, it was in less than 40 % of the rats and spontaneously disappeared.)

significant effects of the 6 mg/kg JMV2959 pretreatment were related to morphine-induced stereotypies, where 80 min after morphine application; there was much less sniffing, and confined gnawing and no licking behaviors were present. During the final 60 min of morphine exposure observations, locomotion was also reduced, but not significantly and immobility significantly increased, while catalepsy remained practically unchanged (6 mg/kg JMV2959 + 5 mg/kg morphine vs. saline+ 5 mg/kg morphine: *locomotion*—effect of group n.s.; effect of time F10,100 = 52.58, $P < 0.001$; time x group interaction n.s.; *stereotypies*—effect of group F1,10 = 117.20, $P < 0.001$; effect of time F10,100 = 58.12, $P < 0.001$; time x group interaction F10,100 = 13.32; *immobility*—effect of group F1, 10 = 30.42, $P < 0.001$; effect of time F10,100 = 39.42, $P < 0.001$; time x group interaction F10,100 = 5.96, $P < 0.001$; *catalepsy*—effect of group n.s.; effect of time F10,100= 314.35, $P < 0.001$; time x group interaction n.s.; two-way ANOVA RM, Bonferroni).

Observations for the acute 10 mg/kg morphine-induced changes with saline and 3 and 6 mg/kg JMV2959 together with saline or ghrelin pretreatment relative to behavioral categories are shown in Fig. 5a–d. The 10 mg/kg morphine-induced catalepsy and immobility lasted longer than after the lower morphine dose, and the stereotypical behavior also lasted longer (120 min after morphine application). The type and spectrum of the JMV2959 6 mg/kg influence on morphine effects was similar to the lower morphine dose: 6 mg/kg JMV2959 + 10 mg/kg morphine vs. saline + 10 mg/kg morphine: *locomotion*—effect of group n.s.; effect of time

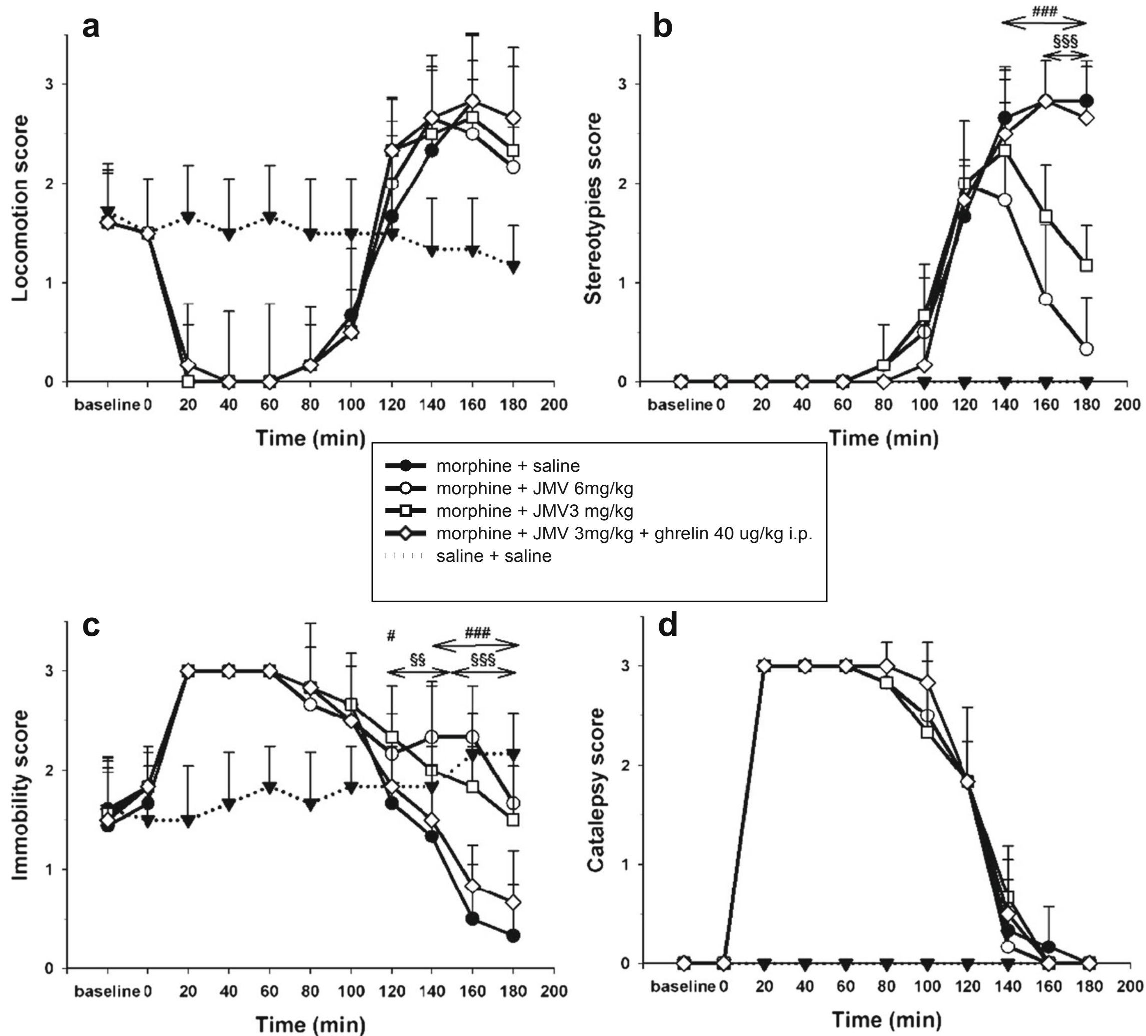

Fig. 5 a–d Effects of GHS-R1A antagonist JMV2959 3 and 6 mg/kg together with saline or ghrelin 40 μg/kg on acute 10 mg/kg MO-induced behavioral changes in four observed categories are illustrated as means of behavioral scores (+/−SEM) separately: (a) locomotion, (b) stereotypies, (c) immobility, and (d) catalepsy. The behavioral effects are illustrated as follows: saline+morphine (*filled circle*), 6 mg/kg JMV2959+morphine (*open circle*), 3 mg/kg JMV2959 with saline+morphine (*open square*), 3 mg/kg JMV2959 together with ghrelin 40 μg/kg+ morphine (*open diamond*), saline+ saline (*filled triangle with dotting*). Differences between morphine and 6 mg/kg JMV2959+morphine or morphine and 3 mg/kg JMV2959+morphine effects are expressed as ###$P<0.001$ or §§§$P<0.001$, respectively. Differences between morphine and 3 mg/kg JMV2959 with ghrelin+ morphine were not significant. All further detailed description is identical with Fig 4 above

$F_{10,100} = 67.12$, $P < 0.001$; time *x* group interaction n.s.; *stereotypies*—effect of group $F_{1,10}=22.96$, $P<0.001$; effect of time $F_{10,100}=59.94$, $P<0.001$; time *x* group interaction $F_{10,100}=15.47$; *immobility*—effect of group $F_{1,10}=13.11$, $P< 0.001$; effect of time $F_{10,100}= 40.45$, $P<0.001$; time *x* group interaction $F_{10,100}=8.66$, $P<0.001$; *catalepsy*—effect of group n.s.; effect of time $F_{10,100}=263.45$, $P<0.001$; time *x* group interaction n.s. The 3 mg/kg JMV2959 effects on 10 mg/kg morphine-induced behavioral changes were similar but less expressed: 3 mg/kg JMV2959 with saline+10 mg/kg morphine vs. saline+10 mg/kg morphine: *locomotion*—effect of group n.s.; effect of time $F_{10,100}=74.07$, $P<0.001$; time *x* group interaction n.s.; *stereotypies*—effect of group $F_{1,10}= 6.40$, $P<0.05$; effect of time $F_{10,100}=100.33$, $P<0.001$; time *x* group interaction $F_{10,100}=8.44$, $P<0.001$; *immobility*—effect of group $F_{1,10}=11.39$, $P<0.01$; effect of time $F_{10,100}= 46.30$, $P < 0.001$; time *x* group interaction $F_{10,100} = 4.71$, $P <0.001$; *catalepsy*—effect of group n.s.; effect of time $F_{10,100}=46.30$, $P<0.001$; time *x* group interaction n.s. Co-administration of ghrelin 40 μg/kg with 3 mg/kg JMV2959 pretreatment abolished the JMV2959 effects; thus, all behavioral parameters at this group did not differ significantly from the saline+ 10 mg/kg morphine group: 3 mg/kg JMV2959 with ghrelin+ 10 mg/kg morphine vs. saline+ 10 mg/kg morphine: the effects of group and time *x* group interaction were n.s. in all four behavioral parameters and the effects of time were as follows: locomotion—$F_{10,100}=74.85$, stereotypies—$F_{10,100}=191.17$, immobility—82.23, and catalepsy—

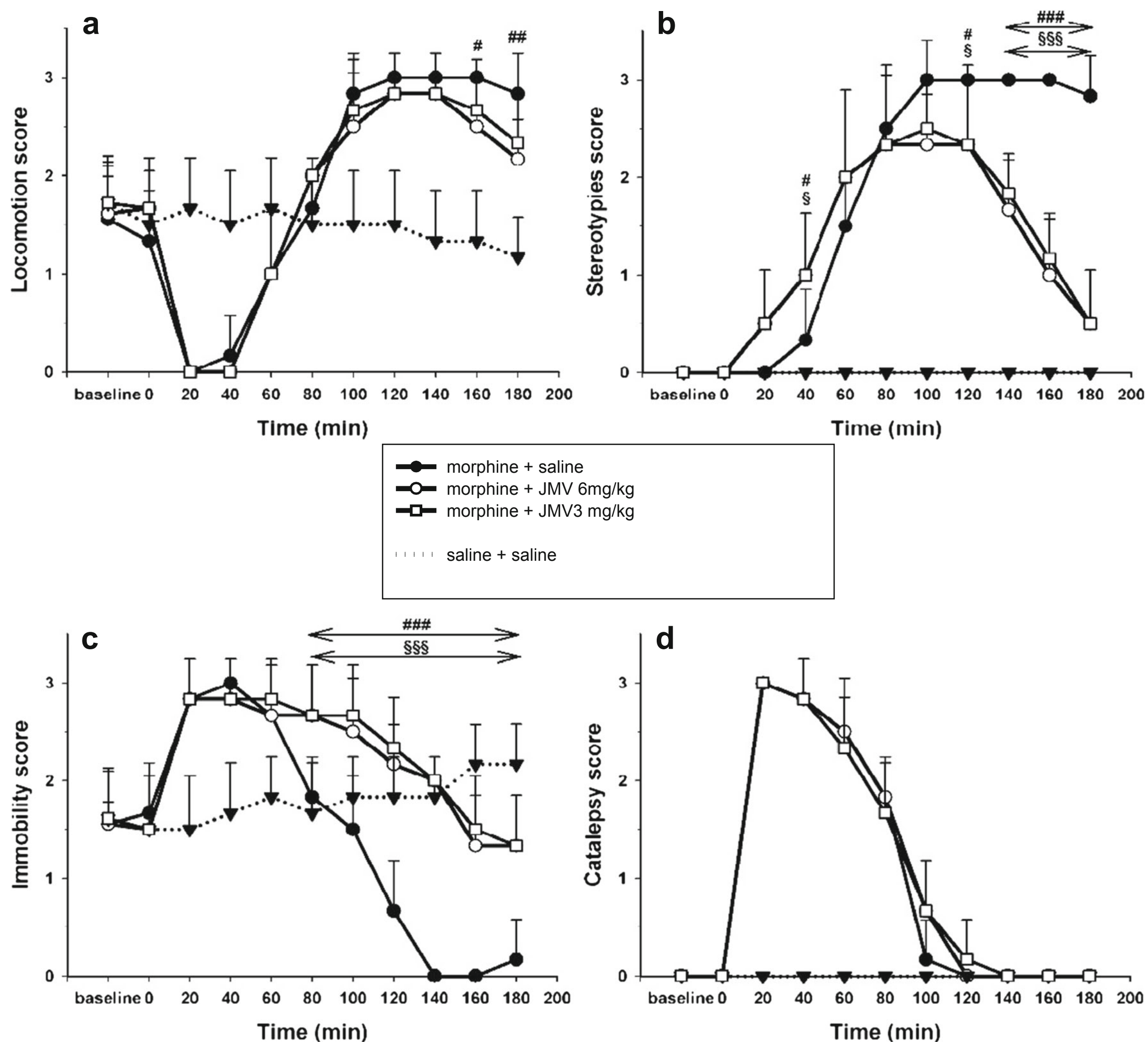

Fig. 6 a–d Effects of GHS-R1A antagonist JMV2959 (3 and 6 mg/kg) on challenge 5 mg/kg MO-induced behavioral changes in four observed categories are illustrated as means of behavioral scores (+/−SEM) separately: (a) locomotion, (b) stereotypies, (c) immobility, and (d) catalepsy. The behavioral effects are illustrated as follows: saline+morphine (*filled circle*), 6 mg/kg JMV2959+morphine (*open circle*), 3 mg/kg JMV2959 with saline+morphine (*open square*), saline+saline (*filled triangle with dotting*). Differences between morphine and 6 mg/kg JMV2959+morphine or morphine and 3 mg/kg JMV2959+morphine effects are expressed as ###P <0.001, ##P <0.01, #P <0.05 or §§§P < 0.001, §P < 0.05, respectively. Further detailed description is identical with Fig. 4 above

123.30. Within the spectrum of stereotypies at this group with co-administered ghrelin, we observed a lot of gnawing similarly to effects of single ghrelin dose (not shown).

The 6 mg/kg JMV2959 alone (graph not shown) produced temporary behavioral changes in less than 40 % of treated rats and always during the first interval (0 min). Typical writhing-like signs could be fully eliminated with touch or sound during the first interval and spontaneously disappeared before morphine, or saline was administered (20 min after 6 mg/kg JMV2959). Similarly, to saline-treated rats, during the last two intervals of dialysis (160–180 min), we observed increased immobility in the 6 mg/kg JMV2959-treated rats: 6 mg/kg JMV2959+saline vs. saline+saline: in all monitored behavioral categories n.s.; occurrence of writhing-like signs only during the first 20 min after JMV2959 application: effect of group $F_{1,10}$=5.00, P=0.049; effect of time $F_{10,100}$=5.00, P<0.001; time x group interaction $F_{10,100}$=5.00 P<0.001; two-way ANOVA RM, Bonferroni.

Morphine administered during un-precipitated abstinence following repeated morphine treatment

Clear signs of behavioral sensitization were observed (increased stereotypical sniffing, gnawing and licking, increased rearing and walking, shortened catalepsy), when 5 mg/kg morphine was given on the 12th day of abstinence following longer-term morphine treatment (N=10 in this rat group). The effects of 6 and 3 mg/kg JMV2959 pretreatment (shown in Fig. 6a–d) seem practically identical, and they are more clearly expressed than in the acute situation. Stereotypical

behaviors and locomotion were significantly reduced, and immobility increased 80–120 min after morphine. For the higher JMV2959 dose: 6 mg/kg JMV2959 + challenge 5 mg/kg morphine vs. saline+ 5 mg/kg MO challenge: *locomotion*—effect of group n.s.; effect of time $F_{10,140}$= 107. 20, P<0.001; time x group interaction $F_{10,140}$=14.32, P<0.05; *stereotypies*—effect of group $F_{1,14}$=29.90, P<0.05; effect of time $F_{10,140}$=65.63, P<0.001; time x group interaction $F_{10,140}$=34.28, P<0.001; *immobility*—effect of group $F_{1,14}$ = 57.03, P < 0.001; effect of time $F_{10,140}$ = 58.24, P <0.001; time x group interaction $F_{10,140}$ = 11.78, P <0.001; *catalepsy*—effect of group n.s.; effect of time $F_{10,140}$= 269.53, P < 0.001; time x group interaction n.s.; two-way ANOVA RM, Bonferroni). For the lower JMV2959 dose: 3 mg/kg JMV2959+challenge 5 mg/kg vs. saline+5 mg/kg MO challenge: *locomotion*—effect of group n.s.; effect of time $F_{10,140}$=116. 25, P<0.001; time x group interaction n.s.; *stereotypies*—effect of group $F_{1,14}$=15.12, P < 0.05; effect of time $F_{10,140}$= 87.43, P < 0.001; time x group interaction $F_{10,140}$=55.52, P<0.001; *immobility*—effect of group $F_{1,14}$=45.23, P<0.001; effect of time $F_{10,140}$= 70.38, P< 0.001; time x group interaction $F_{10,140}$=29.70, P <0.001; *catalepsy*—effect of group n.s.; effect of time $F_{10,100}$= 189.98, P < 0.001; time x group interaction n.s.; two-way ANOVA RM, Bonferroni).

Single 6 mg/kg JMV2959 dose during morphine abstinence induced comparable effects as in the acute situation, the writhing-like signs occurred in less than 40 % of rats and disappeared within the first 20 min after injection (not shown). Application of challenge saline had no significant effects on the rat behavior.

Discussion

Our results for the first time indicated significant involvement of ghrelin signaling in morphine-induced changes in the mesolimbic endocannabinoid AEA and 2-AG extracellular concentrations and in morphine-induced behavioral sensitization.

Endocannabinoid tone is essential for opioid reinforcing properties (see the ʙIntroduction^ for references). In our in vivo microdialysis experiments, acute subcutaneous administration of morphine 5 and 10 mg/kg induced significant and opposing changes in the extracellular endocannabinoid concentrations in the NACSh: dose-dependent increases of AEA levels, with the maximum of 117 and 142 % of baseline mean, respectively, and 2-AG decrease (to maximum of 86 and 85 % of baseline mean) in accordance with Vigano et al. (2004; post mortem NAC tissue). The detected accumbens baseline concentrations of both AEA and 2-AG in naive rats were roughly in accordance with the literature (Felder et al. 1996; Vigano et al. 2004; Caille et al. 2007; Wiskerke et al. 2012).

In the longer-term experiment, the model of sensitization, we measured accumbens endocannabinoid changes during administration of a challenge dose of morphine, on the 12th day of an abstinence period, after withdrawal symptoms had already disappeared. In comparison to the acute experiments, we observed significantly decreased 2-AG accumbens baseline levels before morphine administration, while basal AEA accumbens extracellular concentrations remained unchanged. In comparison with acute administration, the challenge dose of 5 mg/kg morphine induced a significantly deeper 2-AG decrease (75 % of baseline mean) and significantly higher increase in accumbens AEA (maximum of 126 % of baseline mean). These results corroborate published findings of Vigano et al. (2004), with three exceptions—Vigano observed increased basal AEA levels during the morphine abstinence period in comparison to morphine-naive rats, and, as a result, the morphine challenge did not induce any further AEA increases. Additionally, the morphine challenge induced a 2-AG decrease that was milder than that after acute morphine. Nevertheless, Vigano et al. (2004) detected endocannabinoids in post mortem NAC tissues from the morphine sensitization rat model. It has been proposed that postmortem endocannabinoid accumulation can be reflected in analyses of brain tissue endocannabinoid content (Patel et al. 2005). Caille et al. (2007) used in vivo microdialysis of the NACSh during heroin intravenous self-administration (IVSA) in rats and found no differences between AEA and 2-AG baselines in naïve animals and baselines in animals with a previous heroin IVSA history. These different findings suggest that repeated non-contingent bolus drug administration induces effects on brain endocannabinoid levels that could be distinct from those induced by daily limited-access IVSA; possible differences between heroin- and morphine-induced effects should also be considered (Andersen et al. 2007). However, our morphine challenge-induced effects in the NACSh during abstinence were in accordance with Caille et al. (2007) who detected AEA increases and 2-AG decreases induced during heroin IVSA sessions.

The GHS-R1A antagonist pretreatment 20 min before morphine provoked significant changes in all acute and challenge morphine-induced effects in accumbens AEA and 2-AG levels. The AEA morphine-induced increase was completely and dose-dependently reversed by JMV2959 pretreatment leading to a significant AEA decrease in both acute and longer-term experiments. On the contrary, the 2-AG morphine-induced decrease was deepened in the acute but slightly yet significantly reduced in the longer-term experiment. JMV2959 influenced accumbens endocannabinoid morphine-induced changes in various ways: reversal in the case of AEA and intensification (in acute) or reduction (in longer-term experiment) in the case of 2-AG. In our previous study (in preparation), single GHS-R1A agonist ghrelin 40 μg/kg dose administration significantly increased both

accumbens AEA and 2-AG together with gnawing and locomotion. Co-administration of ghrelin with JMV2959 3 mg/kg abolished completely the monitored effects of this GHS-R1A antagonist on the morphine-induced changes. Thus, participation of ghrelin signaling in morphine-induced 2-AG and especially AEA accumbens changes is clearly indicated. It has been suggested, that opioid-induced accumbens shell anandamide increase possibly participate in the opioid reinforcement namely through CB1 receptor-mediated DA-independent process (Caille and Parsons 2003, 2006; Caille et al. 2007). Thus, the observed dose-dependent reversal of opioid-induced accumbens anandamide increase, which was provoked by ghrelin antagonist, indicates an important role of ghrelin signaling in the presumed anandamide involvement in the opioid reinforcing mechanisms. The reinforcing properties of anandamide have been well confirmed (AEA IVSA in squirrel monkeys Justinova et al. 2008).

JMV2959 administration in our study also reduced the increased locomotion and stereotypical behaviors (especially licking, gnawing, and stereotypical sniffing) induced by morphine in acute as well as challenge doses, in the model of sensitization. Involvement of ghrelin system was confirmed by ghrelin co-administration. Also these results suggest that ghrelin signaling is involved in mechanisms related to opioid addiction, similarly to studies investigating ethanol and psychostimulants, in which ghrelin antagonism also attenuated or eliminated the behavioral stimulation (Jerlhag et al. 2010, 2011; Jerlhag and Engel 2011; Wellman et al. 2011) and sensitization (Wellman et al. 2013; Abizaid et al. 2011) induced by these drugs. Behavioral sensitization can be with certain limits used for investigating the incentive motivation underlying drug-seeking behavior. Remarkable overlap has been demonstrated between the neurocircuitry involved in sensitization and reinstatement behaviors (Robinson and Berridge 2003; De Vries et al. 1998; Steketee and Kalivas 2011). Cross-sensitization between opiates and cannabinoids has been described (Pontieri et al. 2001a,b; Cadoni et al. 2001; Singh et al. 2005). Both the CB1 receptor antagonist SR141716A and the opioid receptor antagonist naloxone blocked behavioral cross-sensitization, suggesting that an endocannabinoid or an endorphin tone plays a substantial part in the sensitized response to opiates or cannabinoids, respectively. Vigano et al. (2004) observed that SR141716 reduced expression but not the induction phase of behavioral sensitization and that the accumbens AEA and 2-AG were differently and significantly affected during both phases of morphine sensitization. However, the exact role of endocannabinoids in the behavioral sensitization (including opioid sensitization) has not yet been specified.

For testing of the role of ghrelin signaling in the intrinsic reinforcing effects of morphine/opioids, it is essential to adopt specific models of addiction, such as IVSA or conditioned place preference, and we are actually working on them, using the non-peptidic ghrelin antagonist, in contrast to Maric et al. (2012). The results of our present neurochemical and behavioral study suggest strong involvement of accumbens endocannabinoids, especially AEA, in the neural processes associated with opioid-induced behavioral sensitization and indicate that ghrelin antagonism might play an important role in this sensitization and in the accumbens endocannabinoid/AEA changes presumably related to opioid reinforcement (Caille and Parsons 2003, 2006; Caille et al. 2007; Vigano et al. 2004). Thus, further investigation is warranted to assess, whether ghrelin antagonisms and/or generally substances influencing endocannabinoid levels and action, such as ghrelin antagonists, can be used to avoid opioid-seeking behavior.

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Compliance with ethical standards Procedures involving animals, along with their care, were conducted in compliance with international laws; protocols respected the Guidelines of the European Union Council (86/609/EU) and followed animal care instructions set forth by the National Committee for the Care and Use of Laboratory Animals. Experiments were approved by the Expert Committee for Protection of Experimental Animals at the Third Faculty of Medicine, Charles University in Prague and were performed in accordance with the Animal Protection Act of the Czech Republic (No. 246/1992 Sb).

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ORIGINAL INVESTIGATION

Ghrelin receptor antagonism of morphine-induced accumbens dopamine release and behavioral stimulation in rats

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Abstract

Rationale and objectives Ghrelin, an orexigenic (appetite stimulating) peptide activates binding sites in the ventral tegmental area (a structure linked with the neural reward system) allowing it to participate in reward-seeking behavior. An increasing number of studies over the past few years have demonstrated ghrelin's role in alcohol, cocaine, and nicotine abuse. However, the role of ghrelin, in opioid effects, has rarely been examined. The aim of the present study was to ascertain whether a ghrelin antagonist (JMV2959) was able to inhibit markers of morphine-induced activation of the neural reward system, namely morphine-induced increase of dopamine in the nucleus accumbens and behavioral changes in rats.

Methods We used in vivo microdialysis to determine changes of dopamine and its metabolites in the nucleus accumbens shell in rats following morphine (MO, 5, 10 mg/kg s.c.) administration with and without ghrelin antagonist pretreatment (JMV2959, 3, 6 mg/kg i.p., 20 min before MO). Induced behavioral changes were simultaneously monitored.

Results JMV2959 significantly and dose dependently reduced MO-induced dopamine release in the nucleus accumbens shell and affected concentration of by-products associated with dopamine metabolism: 3-methoxytyramine (3-MT), 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA). JMV2959 pretreatment also significantly reduced MO-induced behavioral stimulation, especially stereotyped behavior.

Conclusions Ghrelin secretagogue receptors (GHS-R1A) appear to be involved in the opioid-induced changes in the mesolimbic dopaminergic system associated with the reward processing.

Keywords Morphine · Ghrelin · Neural reward system · Nucleus accumbens shell · Dopamine · Dopamine metabolites · Stereotyped behavior · Microdialysis

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Introduction

Ghrelin, a powerful stomach-released orexigenic peptide (Kojima et al. 1999), exerts its potent orexigenic and pro-obesity effects not only through metabolic homeostatic regulatory mechanisms but (as has been recently described) also by markedly increasing food reward (Egecioglu et al 2010). Ghrelin mediates food intake via central ghrelin secretagogue receptors (GHS-R1A) (Howard et al. 1996; Zigman et al. 2006). In addition to other effects, ghrelin has recently been reported to also markedly activate mesolimbic dopaminergic pathways, which is critically involved in the reinforcement circuitry of the brain's reward system; in particular, activation of the acetylcholine-dopamine (DA) reward link in the ventral tegmental area (VTA), which probably increases reward-seeking behavior (Naleid et al. 2005; Abizaid et al. 2006; Jerlhag et al. 2006, 2010a; Quarta et al. 2009; Skibicka et al. 2011). Ghrelin participation in the brain reward system indicates that it may increase the incentive value of both natural and artificial rewards and may represent some common supportive element enhancing the search for rewards such as rewarding foods and also drugs of abuse.

An increasing number of animal and human studies have supported the key role of ghrelin in alcohol reward mechanisms and consumption (Wurst et al. 2007; Jerlhag et al. 2009; Addolorato et al. 2009). Several studies have described the

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role of ghrelin in the psychostimulants reward system, examples include cocaine (Davis et al. 2007; Tessari et al. 2007; Jerlhag et al. 2010b; Abizaid et al. 2011; Clifford et al 2012), nicotine (Jelhag and Engel 2011; Wellman et al. 2011; Ypsilantis et al. 2013), and amphetamine (Jerlhag et al. 2010b; Liu et al. 2013).

The relationship between ghrelin and opioid dependence has thus far received little attention (Maric et al. 2012; D'Cunha et al. 2013). As with other addictive drugs, activation of the morphine (MO) neural reward pathways is associated with MO-induced increase of DA in the nucleus accumbens (Pothos et al. 1991; Leone et al. 1991; De Vries and Shippenberg 2002; Hyman et al. 2006; Koob and Volkow 2010). MO also induces typical stereotyped behavior changes (Wise and Bozarth 1987; Fiserova et al 1999; Koob and Volkow 2010). A role of ghrelin in the MO-induced increase of DA in the nucleus accumbens, which is believed to be crucial in opioid reward and dependence, has not been studied so far.

Therefore, the aim of the present study was to ascertain whether a systemic application of the ghrelin antagonist JMV2959 was able to inhibit the MO-induced changes in the mesolimbic DA system, and in the concomitant behavioral changes. In order to get a more complete picture, we also monitored DA metabolism in the nucleus accumbens.

Materials and methods

Animals

Male Wistar rats (Velaz, Anlab Czech Republic), approximately 8 weeks old, weighing 200–240 g were used. The animals were given free access to water and food and were housed in polycarbonate cages with constant humidity (50–60 %), room temperature (22–24 °C), and a 12-h light/dark cycle, for at least 7 days before the experiments, which were performed from 8 a.m. to 3 p.m. Groups of six rats were used for each treatment. Procedures involving animals, along with their care, were conducted in compliance with international laws; protocols respected the Guidelines of the European Union Council (86/609/EU) and followed the instructions of the National Committee for the Care and Use of Laboratory Animals. Experiments were approved by the Expert Committee for Protection of Experimental Animals of the Third Faculty of Medicine, Charles University, Prague and were performed in accordance with the Animal Protection Act of the Czech Republic (No. 246/1992 Sb).

Drugs and chemicals

All reagents were analytical grade. MO hydrochloride was purchased from Dr. Kulich Pharma (CR). JMV2959 (1,2,4-triazole derivate), which has been demonstrated to be an GHS-R1A antagonist (Moulin et al. 2007), was kindly provided by Anton Bespalov, AbbVie, Germany. Both substances were dissolved in saline, and saline was used as a placebo. MO (5 or 10 mg/kg) was administered subcutaneously (s.c.) in volumes of 0.1–0.2 ml/100 g of body weight. The selected doses of JMV2959 (3 and 6 mg/kg) were determined based on the literature (Jerlhag et al. 2010b; Clifford et al. 2012) and on the results of our previous pilot study with Wistar rats. The higher JMV2959 dose (6 mg/kg) produced temporary behavioral changes in less than 40 % of treated rats, which could be fully eliminated with sound or touch and spontaneously disappeared within 20 min after administration. JMV2959 was administered intraperitoneally (i.p.) 0.1 ml/100 g of body weight and always 20 min prior to MO or saline injections.

Schedule of experiments

Acute effects of MO, in rats, after pretreatment with JMV2959 (3 or 6 mg/kg) or saline were monitored using in vivo microdialysis of the nucleus accumbens shell (NACSh). Simultaneously with dialysis, the same animals were monitored for MO-induced behavioral changes. Treatment groups were as follows: saline + saline; saline + MO 5 mg/kg; saline + MO 10 mg/kg; JMV2959 6 mg/kg + saline; JMV2959 6 mg/kg + MO 5 mg/kg; JMV2959 6 mg/kg + MO 10 mg/kg; JMV2959 3 mg/kg + MO 10 mg/kg. The dialysis samples were collected at 20 min intervals for a total of 260 min. Dialysates were analyzed for the concentration of DA and its metabolites (3-methoxytyramine (3-MT), 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA)) using high-sensitivity liquid chromatography combined with mass spectrometry.

In vivo microdialysis: assay of dopamine and its metabolites

Surgery

Under ketamine–xylazine anesthesia (ketamine 100 mg/kg i.p., Narketan 100 mg/ml Vetoquinol; xylazine 10 mg/kg i.p., Xylapan 20 mg/ml, Vetoquinol), rats were implanted with a disposable dialysis guide cannula (MAB4 probes, Agnthos, Stokholm, Sweden) using a stereotaxic instrument (StoeltingCo). After taking the co-ordinates with a guide mounted on the stereotaxic holder (NACSh: A: +2.0 mm and L: ±1.2 mm from bregma and V: 6.2 mm from occipital bone) (Paxinos and Watson 1986), the guide was slowly lowered into the brain and secured to the skull with dental cement and an anchoring screw. The guide was randomly alternated on the left and right side. After surgery, the rats were kept in individual cages. After completion of the microdialysis experiments, the placement of the dialysis probe

was verified histologically (Fig. 1). Animals with the probe outside the shell region were excluded from the experiment.

Microdialysis and chemical analysis assay

At least 48 h after implantation, the probe (MAB4, 2 mm active cuprophane membrane, Agnthos, Stokholm, Sweden) was inserted into the guide cannula and artificial cerebrospinal fluid (ACSF; Ringer's solution; 147 mM NaCl, 2. 2 mM CaCl2, and 4.0 mM KCl; adjusted to pH 7.0) was flushed through the probe at a constant rate of 2.0 μl/min (Univentor 864 Syringe Pump, Agnthos, Stokholm, Sweden). After 40 min of habituation to the microdialysis setup (when dialysate was discarded), 20 μl samples were collected at 20-min intervals in small polyethylene test tubes containing 7 μl HCl 0.1 mM, to prevent catecholamine hydrolysis. After three consecutive baseline samples, rats were injected with saline or JMV2959 (i.p.), which was followed (20 min later) by MO or were collected for 3 h following injection of MO or saline. Immediately following collection, the samples were frozen at −70 °C. The amount of DA and its metabolites (3-MT, DOPAC, and HVA) in the dialysate were quantified using HPLC-MS. Determination methods for DA and its metabolites, in ACSF, consisted of a pretreatment step, freeze drying (lyophilization) to concentrate the substances from the microdialysates, and a detection step (consisting of liquid chromatography combined with electrospray ionization tandem mass spectrometry (LC−ESI-MS/MS)). The frozen ACSF sample (−80 °C) with artificially added internal standard (IS), i.e., deuterium labeled DA (DA-d_4 10 pg) was inserted into a freeze dryer (Labconco Free Zone, USA) for 2 h. The freeze dryer condenser coil was cooled to −47 °C, and the device pressure was maintained at 9 kPa. The lyophilization residue was dissolved while vortexing in methanol (50 μl), which led to precipitation of residual salts, peptides, etc. A suspension of precipitated salts was centrifuged (2 min; 700 g), and the supernatant was immediately analyzed using LC−ESI-MS/MS. The LC-MS system consisted of a chromatograph Accela 1250 (Thermo Scientific, USA), autosampler Accela (Thermo Scientific, USA), and a TSQ Vantage mass spectrometer (Thermo Scientific, USA). The analytes were separated on a Gemini® NX-C18 (5 μm at 110 Å) LC column (150 mm × 2 mm) using a mobile phase (solvent A: aqueous solution of acetic acid (pH 2); solvent B: methanol) with a gradient elution flow rate of 150 μl/min. The HPLC elution program was set up as follows: 5 % B (3 min) → 30 % B (linear increase in 2 min) → 30 % B (10 min) → 5 % B (linear decrease in 1 min) → 5 % B (4 min). The column temperature was maintained at 25 °C. The injection volume was 5 μl. The mass spectrometer, equipped with an electrospray ionization source, was used to detect DA. A deuterium labeled IS DA-d_4, and 3-MT operated in positive ionization mode (ESI$^+$) and HVA, DOPAC operated in negative ionization mode (ESI$^-$). A selective reaction monitoring (SRM) mode was used. Scan monitoring reactions (precursor ion → fragment ion) were used for the analyses and their collision-induced dissociated (CID) energy were as follows: m/z = 137 → 120 (CID = 17.5 eV) for DA, m/z = 141 → 124 (CID = 17 eV) for DA-$d4$, m/z = 181 → 122 (CID = 17.0 eV) for HVA, m/z = 168 → 151 (CID = 11.5 eV) for 3-MT, and m/z = 167 → 122 (CID = 8.5 eV) for DOPAC. The conditions on the mass spectrometer were optimized and were as follows: spray voltage 3,000 V, temperature of ion transfer tube 350 °C, temperature of H-ESI vaporizer 350 °C, sheath gas pressure (nitrogen) 35 psi, flow of auxiliary gas (nitrogen) 10 arb. units.

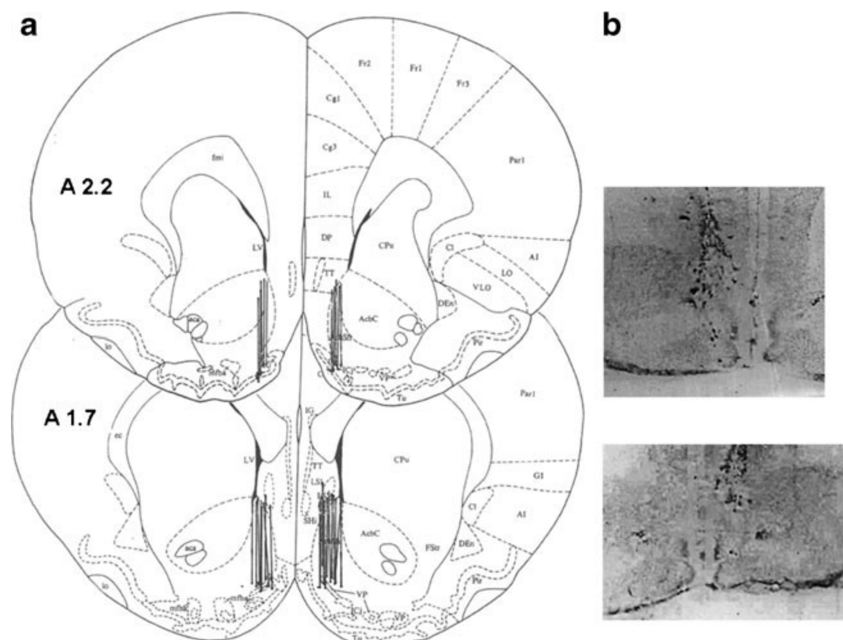

Fig. 1 Location of dialysis probes within the nucleus accumbens shell (NACSh). a Schematic locations of probe tips in rats, which were included in analyses of accumbens neurotransmitter concentrations (the *solid lines* indicate the dialyzing portions) as described in the atlas of Paxinos and Watson (1986). On the left, for each section, the distance from bregma (in millimeter) is indicated. b Representative photomicrographs showing dialysis probe placements in the NACSh (only most ventral cannula track contains active dialysis membrane)

The data were acquired and processed using Xcalibur 2.1.0 software (Thermo Scientific, USA). A detailed description of methods can be found in a study by Syslová et al. (2011).

Behavioral assay

Behavior was studied simultaneously, in the same animals, while microdialysis measurements were being performed. The following behavioral categories were distinguished as follows: immobility (sedation, eyes closed, akinesia, and reduced responsiveness to environmental cues), catalepsy (frozen postures, exophthalmos, and trunk rigidity), locomotion (non-stereotyped activity, sniffing, grooming, rearing, and walking), stereotyped activity (confined gnawing, licking, and stereotypical sniffing), and other symptoms (writhing) (Fiserova et al. 1999; Acquas and Di Chiara 1992; Rada et al. 1996). Behavioral categories were scored every 20 min (at each microdialysis interval) by an observer who was unaware of the treatment each rat had received. The percentage of time spent by the animal in each behavioral category was calculated for each 20-min interval. Behavioral changes were monitored during the entire dialysis period (60 min baseline, 20 min pretreatment, and 3 h following MO or saline injection).

Statistical analysis

Raw data for DA and its metabolites (expressed as picogram per milliliter per sample or nanogram per milliliter per sample, not corrected for probe recovery) were transformed into a percentage of baseline levels (mean of the three values prior to pretreatment). The behavioral parameters during 20-min intervals were also analyzed (converted to percentages). Time course neurochemical and behavioral data were statistically analyzed (SigmaStat 3.5, Systat Software, Inc., USA) using a one-way analysis of variance (ANOVA) for repeated measures (RM) followed by a multiple comparison Bonferroni's test to compare all treatments to baseline or using a two-way ANOVA with a between-subjects factor relative to treatment and a repeated measurements factor for time (20-min intervals over 200 min of posttreatment) followed by a multiple comparison Tukey's test or Bonferroni's test (for behavior). P values of <0.05, <0.01, and <0.001 defined statistical significance. Results are presented as the mean ± standard error *of the* mean (SEM).

Results

The effects of GHS-R1A antagonist on morphine-induced accumbens DA release

The influence of ghrelin antagonism on MO-induced DA release in the nucleus accumbens is illustrated in Fig. 2a, b. DA baseline levels did not differ significantly between animals. As expected, MO induced a dose-dependent, statistically significant, increase of DA release in the NACSh. The lower MO dose (5 mg/kg s.c.) was significantly effective with the maximum DA increase occurring 60 min postadministration (174 % of baseline level) (saline + MO 5 mg/kg: $F_{10,49} = 39.10$, $P < 0.001$; one-way ANOVA for RM followed by Bonferroni's test versus baseline). The larger MO dose (10 mg/kg s.c.) effect reached its maximum again

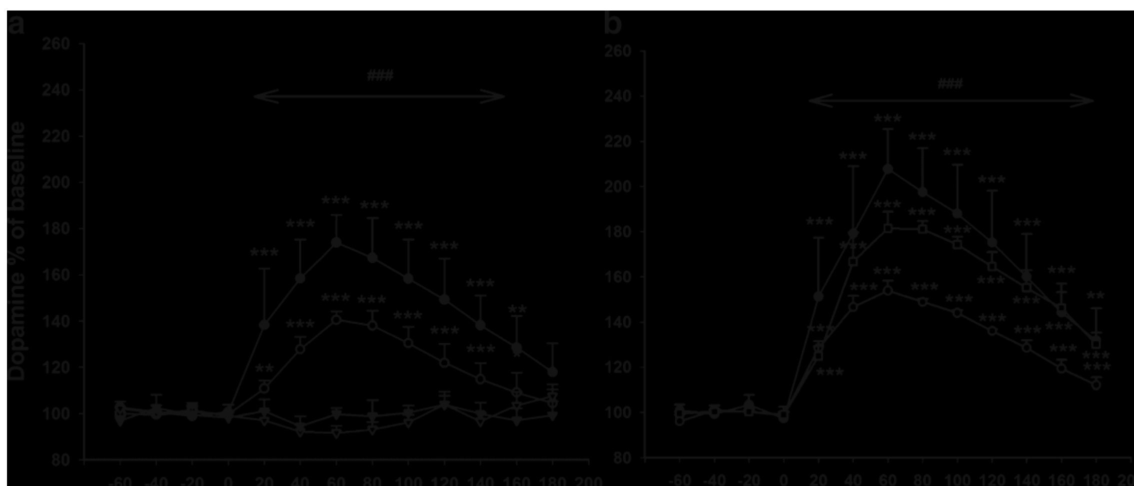

Fig. 2 The effects of ghrelin receptor (GHS-R1A) antagonist (JMV2959) on MO-induced dopamine release in the rat NACSh. JMV2959 was always given i.p. 20 min before MO/saline s.c. injection (*n* = *6 for all groups*; *means±SEM*). The effects are illustrated as follows: a saline+MO 5 mg/kg (*filled circle*), JMV2959 6 mg/kg+MO 5 mg/kg (*open circle*), JMV2959 6 mg/kg+saline (*open triangle*), saline+saline (*filled triangle*). b saline+MO 10 mg/kg (*filled circle*), JMV2959 6 mg/kg+MO 10 mg/kg (*open circle*), JMV2959 3 mg/kg+MO 10 mg/kg (*open square*). Differences between treatments and baseline mean are expressed as $^{***}P<0.001$, $^{**}P<0.01$, the differences between single MO versus JMV2959 6 mg/kg pretreated MO effects are expressed as $^{###}P<0.001$. (At $P<0.05$, the differences between MO and JMV2959 3 mg/kg+MO effects were not significant.)

60 min postadministration (208 % of baseline level) (saline + MO 10 mg/kg: $F10,49 = 61.28$, $P < 0.001$; one-way ANOVA RM, Bonferroni).

Pretreatment with the GHS-R1A antagonist JMV2959 6 mg/kg reduced the 5 mg/kg MO-induced increase in DA to a maximum of 140 % of baseline, which represented significant reduction (differences between drugs $F10,104 = 2.23$, $P < 0.05$; two-way ANOVA followed by Tukey's test); however, the MO-induced DA increase still remained significant (JMV2959 + MO 5 mg/kg: $F10,49 = 59,35$, $P < 0.001$; one-way ANOVA RM, Bonferroni). At the higher MO dose (10 mg/kg), pretreatment with JMV2959 6 mg/kg reduced the MO-induced DA to a maximum of 154 % of baseline (differences between drugs $F10,104 = 4.17$, $P < 0.001$; two-way ANOVA, Tukey); as before, the MO-induced DA increase remained significant (JMV2959 6 mg/kg + MO 10 mg/kg: $F10,49 = 215.19$, $P < 0.001$; one-way ANOVA RM, Bonferroni). Pretreatment with JMV2959 3 mg/kg i.p. slightly but not significantly reduced the 10 mg/kg MO-induced increase in DA; thus, again the DA increase remained significant (JMV2959 3 mg/kg + MO 10 mg/kg: $F10,49 = 149.48$, $P < 0.001$; one-way ANOVA RM, Bonferroni). A single dose of JMV2959 (6 mg/kg i.p.) did not change the DA output in the NACSh; the same was also true for saline.

The effects of a GHS-R1A antagonist on MO-induced increase of DA metabolites in the NACSh

The influence of ghrelin antagonism on acute MO-induced DA metabolism (measured in the NACSh) is illustrated in Fig. 3a–f. As expected, we found significant MO-induced dose-dependent increases of DOPAC, 3-MT, and HVA, at both MO doses. The effects of JMV2959 pretreatment were variable; however, the total accumbens DA metabolism (HVA concentration) was slightly and transiently increased.

3-MT

For 3-MT (see Fig. 3a, b), the lower MO dose induced significant increase with a maximum 60 min post-MO injection (137 % of baseline) (saline + MO 5 mg/kg: $F10,49 = 45.88$, $P < 0.001$; one-way ANOVA RM, Bonferroni). The larger MO dose induced significant 3-MT increase with the maximum occurring 60 min post-MO injection (147 % of baseline) (saline + MO 10 mg/kg: $F10,49 = 38.87$, $P < 0.001$; one-way ANOVA RM, Bonferroni).

Pretreatment with JMV2959 6 mg/kg significantly attenuated increase in 3-MT concentrations (in the NACSh) associated with the 5 mg/kg MO injection (differences between drugs $F10,104 = 2.65$, $P < 0.05$; two-way ANOVA, Tukey). The maximum effect was postponed to 80 min post-MO injection, and the maximum dropped to 123 % of baseline; the 3-MT increase still remained significant (JMV2959 + MO 5 mg/kg: $F10,49 = 68.43$, $P < 0.001$; one-way ANOVA RM, Bonferroni). At 10 mg/kg, both JMV2959 doses (3 and 6 mg/kg) did not significantly influence MO-induced 3-MT increases, the 10 mg/kg MO effects remained almost the same as in non-pretreated situation. A single dose of JMV2959 (6 mg/kg i.p.) did not change the concentration of 3-MT, and the same was true for saline.

DOPAC

The effects of GHS-R1A antagonist (JMV2959) on MO-induced DOPAC formation in the accumbens are illustrated in Fig. 3c, d. The 5 mg/kg MO dose induced significant DOPAC increase with the maximum, 127 % of baseline, occurring 60 min post-MO injection (saline + MO 5 mg/kg: $F10,49 = 38.00$, $P < 0.001$; one-way ANOVA RM, Bonferroni). The 10 mg/kg MO dose induced significant DOPAC increase with a maximum 80 min post-MO injection (131 % of baseline) (saline + MO 10 mg/kg: $F10,49 = 42.55$, $P < 0.001$; one-way ANOVA RM, Bonferroni).

JMV2959 6 mg/kg pretreatment did not significantly influence the 5 mg/kg MO-induced DOPAC increase. However, pretreatment with JMV2959 significantly dose dependently augmented the 10 mg/kg MO-induced DOPAC increase (JMV2959 at 6 mg/kg: differences between drugs $F10,104 = 6.62$, $P < 0.001$; JMV2959 at 3 mg/kg: differences between drugs $F10,104 = 2,69$, $P < 0.01$; two-way ANOVA, Tukey). Following pretreatment with JMV2959 6 mg/kg, the maximum effect was intensified to 153 % of baseline, 60 min post-MO (JMV2959 6 mgk/kg + MO 10 mg/kg: $F10,49 = 214.89$, $P < 0.001$; one-way ANOVA RM, Bonferroni). Following pretreatment with 3 mg/kg JMV2959, the maximum effect was intensified to 141 % of baseline, 80 min post-MO (JMV2959 3 mg/kg + 10 mg/kg: $F10,49 = 149.48$; $P < 0.001$; one-way ANOVA RM, Bonferroni). A single dose of JMV2959 (6 mg/kg i.p.) slightly (temporarily significantly) augmented the increase in DOPAC, maximum 108 % of baseline, 60 min postinjection (JMV2959 + saline: $F10,49 = 17.56$, $P < 0.001$; one-way ANOVA RM, Bonferroni). Application of saline had no effect on DOPAC.

HVA

The effects of GHS-R1A antagonist on MO-induced HVA increase in the NASh are illustrated in Fig. 3e, f. The lower MO dose induced significant HVA increase with a maximum 80-min post-MO injection (124 % of baseline) (saline + MO 5 mg/kg: $F10,49 = 40.45$, $P < 0.001$; one-way ANOVA RM, Bonferroni). The larger MO dose was significant with the maximum HVA increase occurring 80-min post-MO injection (139 % of baseline) (saline + MO 10 mg/kg: $F10,49 = 42.58$, $P < 0.001$; one-way ANOVA RM, Bonferroni).

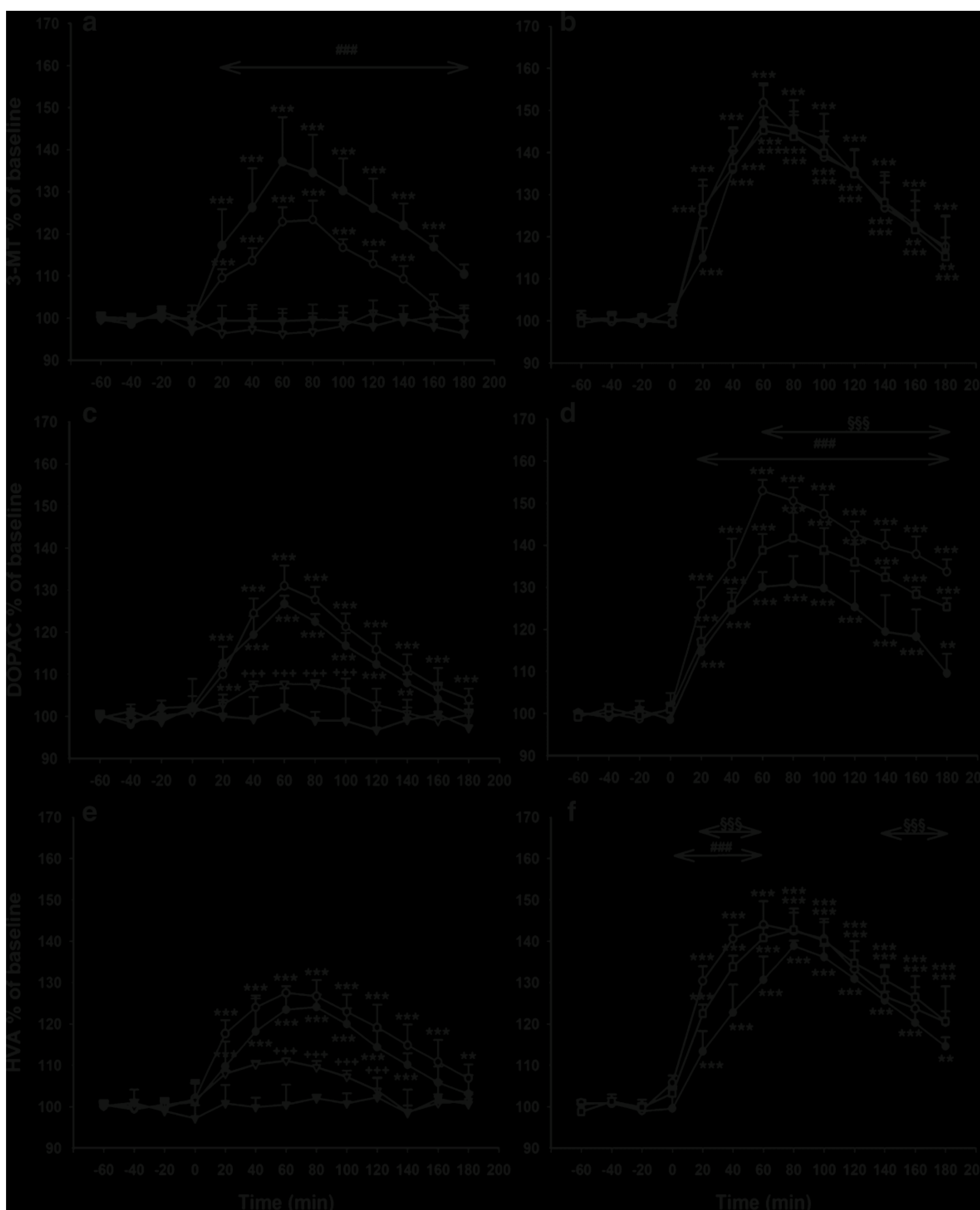

Fig. 3 Effects of a ghrelin GHS-R1A antagonist (JMV2959) administration on the dopamine turnover (ergo the 3-MT, DOPAC and HVA concentrations) in the NACSh after a single morphine dose (MO) s.c. administered to rats (*n = 6 for all groups*; *means±SEM*); JMV2959 was always applied i.p. 20 min before MO/saline s.c. injection. The effects on accumbens metabolites are illustrated as follows: a effects on 3-MT: saline+MO 5 mg/kg (*filled circle*), JMV2959 6 mg/kg+MO 5 mg/kg (*open circle*), JMV2959 6 mg/kg+saline (*open triangle*), saline+saline (*filled triangle*); b effects on 3-MT: saline+MO 10 mg/kg (*filled circle*), JMV2959 6 mg/kg+MO 10 mg/kg (*open circle*), JMV2959 3 mg/kg+MO 10 mg/kg (*open square*); c effects on DOPAC: saline+MO 5 mg/kg (*filled circle*), JMV2959 6 mg/kg+MO 5 mg/kg (*open circle*), JMV2959 6 mg/kg+saline (*open triangle*), saline+saline (*filled triangle*); d effects on DOPAC: saline+MO 10 mg/kg (*filled circle*), JMV2959 6 mg/kg+MO 10 mg/kg (*open circle*), JMV2959 3 mg/kg+MO 10 mg/kg (*open square*); e effects on HVA: saline+MO 5 mg/kg (*filled circle*), JMV2959 6 mg/kg+MO 5 mg/kg (*open circle*), JMV2959 6 mg/kg+saline (*open triangle*), saline+saline (*filled triangle*); and f effects on HVA: saline+MO 10 mg/kg (*filled circle*), JMV2959 6 mg/kg+MO 10 mg/kg (*open circle*), JMV2959 3 mg/kg+MO 10 mg/kg (*open square*). Differences between treatments and baseline mean are expressed as $^{***}P<0.001$, $^{**}P<0.01$, or $^{+++}P<0.001$, $^{++}P<0.01$, respectively. Differences between MO and JMV2959 6 mg/kg +MO or MO and JMV2959 3 mg/kg+MO effects are expressed as $^{\#\#\#}P<0.001$ or $^{§§§}P<0.001$, respectively

JMV2959 pretreatment did not significantly influence the 5 mg/kg MO-induced HVA increase, and the observed increase remained significant with a maximum occurring 60 min post-MO (127 % of baseline) (JMV2959 + MO 5 mg/kg: $F_{10,49} = 104.52$, $P < 0.001$; one-way ANOVA RM, Bonferroni). Pretreatment with JMV2959 6 mg/kg temporarily significantly augmented the 10 mg/kg MO-induced HVA increase (differences between drugs $F_{10,104} = 4.45$, $P < 0.001$; two-way ANOVA, Tukey). The maximum effect increased to 144 % of baseline (60 min post-MO) (JMV2959 6 mg/kg + MO 10 mg/kg: $F_{10,49} = 72.47$, $P < 0.001$; one-way ANOVA RM, Bonferroni). Also pretreatment with JMV2959 3 mg/kg temporarily augmented the 10 mg/kg MO-induced HVA increase (differences between drugs $F_{10,104} = 2.04$, $P < 0.05$; two-way ANOVA, Tukey), with maximum increase to 141 % of baseline (80 min post-MO) (JMV2959 3 mg/kg + MO 10 mg/kg: $F_{10,49} = 118.23$, $P < 0.001$; one-way ANOVA RM, Bonferroni). A single dose of JMV2959 (6 mg/kg i.p.) slightly (temporarily significantly) increased HVA concentrations, with a maximum of 111 % of baseline level (JMV2959 + saline: $F_{10,49} = 17.70$, $P < 0.001$; one-way ANOVA RM, Bonferroni). Application of saline alone did not change HVA concentrations.

The effects of GHS-R1A antagonist on morphine-induced behavioral changes

Observed biphasic (inhibition-stimulation) MO-induced changes in rats during all microdialysis experiments (except the group of rats treated with JMV2959 3 mg/kg + 10 mg/kg MO) are illustrated in Fig. 4a–d. The behavioral changes are shown with respect to baseline conditions (mean of 60 min = three 20-min intervals of baseline period—baseline control values); 0 min illustrates the time interval after pretreatment (with JMV2959 6 mg/kg or saline), followed by 20–180 min of the MO or saline effect.

Statistically significant effects of 5 mg/kg MO dose were visible in all evaluated behavioral parameters during the marked intervals (Fig. 4a) (saline + MO 5 mg/kg: immobility: $F_{10,50} = 47.61$ /20–60 min, 100–180 min/; catalepsy:

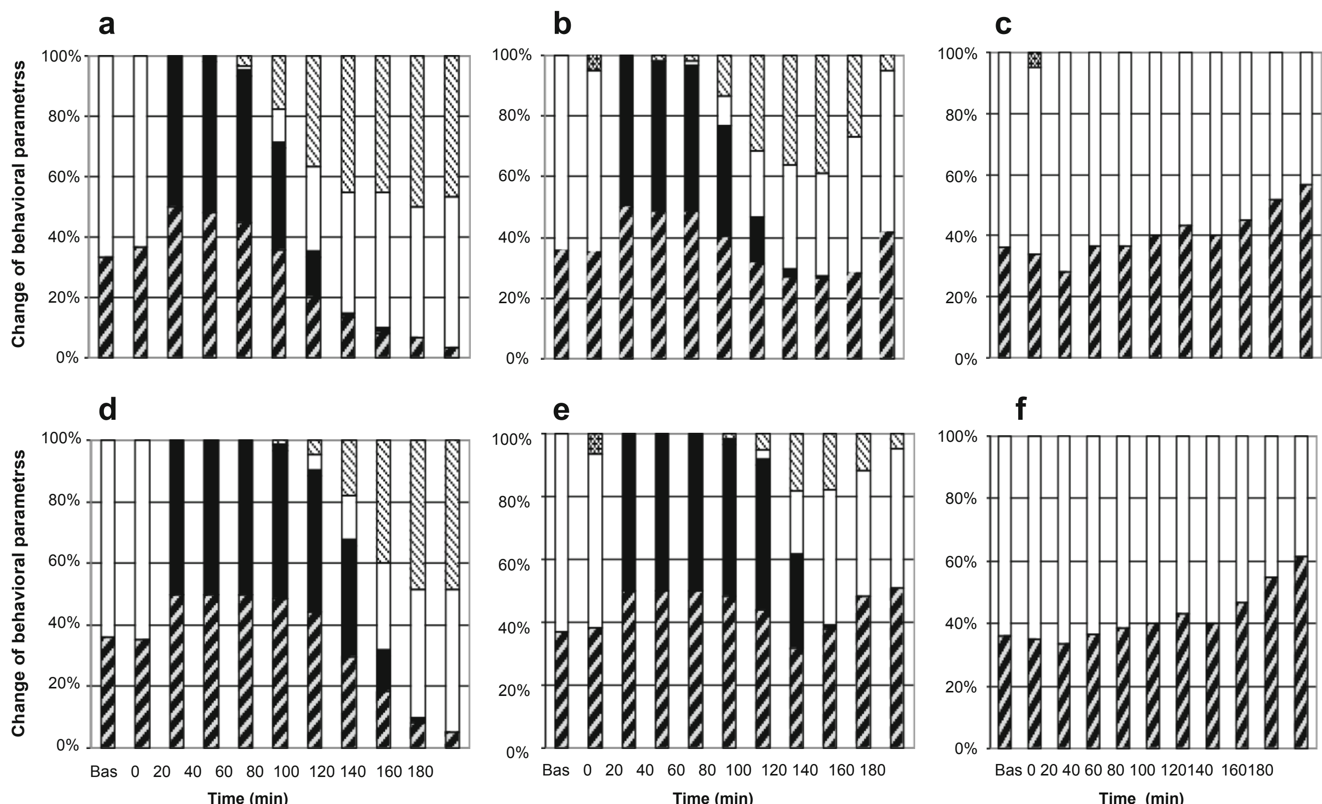

Fig. 4 The effects of GHS-R1A antagonist on morphine (MO)-induced behavioral changes in five different observed parameters: immobility (*thick diagonal strips*), catalepsy (*black*), locomotion (*white*), stereotyped activity (*thin diagonal strips*), and others (*grid pattern*) are illustrated as a percentage of total behavior during each 20 min interval (total behaviors = 100 %) (the means from *n*=6 rats are presented). JMV2959 was applied i.p. 20 min before MO/saline. The behavioral changes are shown with respect to baseline conditions (mean of 60 min of baseline period) (*Bas = control*), the time interval after pretreatment with JMV2959/saline (0), is followed by 20–180 min of the MO/saline effects (*20–180 min*). The results were obtained during dialysis in rats treated with the following: a saline+MO 5 mg/kg, b JMV2959 6 mg/kg+MO 5 mg/kg, c JMV2959 6 mg/kg+saline, d saline+MO 10 mg/kg, e JMV2959 6 mg/kg+MO 10 mg/kg, f saline+saline. (The behavioral changes in rats treated with JMV2959 3 mg/kg+MO 10 mg/kg are not shown.)

$F_{10,50}$ = 107.60 /20–100 min/; locomotion: $F_{10,50}$ = 95.76 / 20–180 min/; stereotyped activity: $F_{10,50}$ = 109.05 /80–180 min/, in all cases $P < 0.001$; one-way ANOVA RM, Bonferroni's test versus control). The initial MO inhibitory effect (immobility and catalepsy) during the 0–80-min time interval was followed by marked behavioral stimulation (increased locomotion with significant stereotypes), which lasted until the end of the experiment. The 10 mg/kg MO dose (Fig. 4d) also induced statistically significant changes (saline + MO 10 mg/kg: immobility: $F_{10,50}$ = 55.77 /20–80 min, 120–180 min/; catalepsy: $F_{10,50}$ = 163.68 /20–140 min/; locomotion: $F_{10,50}$ = 144.25 /20–180 min/; stereotyped activity: $F_{10,50}$ = 152.26 /120–180 min/, in all cases $P < 0.001$; one-way ANOVA RM, Bonferroni's test versus control), with much longer behavioral inhibition (which lasted about 2 h). During the 120 min interval, the mode of behavior slowly started to change to excitatory signs (locomotion, significant stereotyped activity after 120 min). Marked stimulation was seen during the last three intervals.

Pretreatment with 6 mg/kg JMV2959, significantly reduced the behavioral stimulation induced by MO (at both MO doses); the biphasic effect remained (JMV2959 6 mg/kg + MO 5 mg/kg: immobility: $F_{10,50}$ = 12.03, /40–60 min/; catalepsy: $F_{10,50}$ = 181.58 /20–100 min/; locomotion: $F_{10,50}$ = 74.91 /20–180 min/; stereotyped activity: $F_{10,50}$ = 109.05 / 80–160 min/, in all cases $P < 0.001$; one-way ANOVA RM Bonferroni's test versus control) (JMV2959 6 mg/kg + MO 10 mg/kg: immobility: $F_{10,50}$ = 7.58 /20–80 min, 160–180 min/; catalepsy: $F_{10,50}$ = 476.72 /20–120 min/; locomotion: $F_{10,50}$ = 164.56 /20–180 min/; stereotyped activity: $F_{10,50}$ = 11.61 /120–160 min/, in all cases $P < 0.001$; one-way ANOVA RM, Bonferroni's test versus control).

The JMV2959 6 mg/kg administration before the 5 mg/kg MO dose (Fig. 4b) significantly reduced manifestations of stereotypical behavior (much less sniffing, less confined gnawing, and no licking) and shorten it by the last time interval (differences between drugs $F_{10,110}$ = 20.93 /120–180 min/, $P < 0.001$, two-way ANOVA Bonferroni). Immobility behavior was significantly increased during the last 1.5 h of dialysis (differences between drugs $F_{10,110}$ = 10.64 /100–180 min/, $P < 0.001$, two-way ANOVA, Bonferroni). The 6 mg/kg JMV2959 pretreatment of the 5 mg/kg MO did not significantly influence MO-induced catalepsy and locomotion; while not significant, we observed less rearing and walking.

The 6 mg/kg JMV2959 administration before the 10 mg/kg MO dose (Fig. 4e) similarly significantly reduced and shortened stereotypical behavior (differences between drugs $F_{10,110}$ = 33.43 /140–180 min/, $P < 0.001$, two-way ANOVA, Bonferroni) together with significant enhancement of immobility during the same time (differences between drugs $F_{10,110}$ = 22.29 /140–180 min/, $P < 0.001$, two-way ANOVA, Bonferroni). Also the 10 mg/kg MO-induced catalepsy was significantly reduced and shortened by 6 mg/kg JMV2959 pretreatment (differences between drugs $F_{10,110}$ = 2.59 /120–140 min/, $P < 0.001$, two-way ANOVA, Bonferroni), and, because of reduced stereotypical behaviors, locomotion became significantly greater during the 140 min time interval (differences between drugs $F_{10,110}$ = 2.93 / 140 min/, $P < 0.001$, two-way ANOVA, Bonferroni).

The JMV2959 3 mg/kg before 10 mg/kg MO dose (graph not shown) also significantly reduced stereotypical behaviors (especially licking and sniffing), but the JMV2959 effects were less expressed than after the 6 mg/kg dose administration (differences between drugs $F_{10,110}$ = 19.73 /140–180 min/, $P < 0.001$, two-way ANOVA, Bonferroni). Immobility was proportionally enhanced at the same intervals (differences between drugs $F_{10,110}$ = 11.98 /140–180 min/, $P < 0.001$, two-way ANOVA, Bonferroni). Also by 3 mg/kg JMV2959 pretreatment, the MO-induced catalepsy was reduced (differences between $F_{10,110}$ = 2.59 /120–140 min/, $P < 0.05$, two-way ANOVA, Bonferroni) and locomotion became significantly greater during the 140-min time interval (differences between drugs $F_{10,110}$ = 1.9 /140 min/, $P < 0.05$, two-way ANOVA, Bonferroni). The biphasic (inhibition-stimulation) MO-induced effect remained (JMV2959 3 mg/kg + MO 10 mg/kg: immobility: $F_{10,50}$ = 4.04 /20–60 min/, $P < 0.05$; catalepsy: $F_{10,50}$ = 176.72 /20–120 min/, $P < 0.001$; locomotion: $F_{10,50}$ = 120.63 /20–180 min/, $P < 0.001$; stereotyped activity: $F_{10,50}$ = 17.53 /120–180 min/, $P < 0.001$; one-way ANOVA RM, Bonferroni's test versus control). The 3 mg/kg JMV2959 alone did not induce any effects significantly different from saline.

The JMV2959 alone, at 6 mg/kg (Fig. 4c), produced temporary behavioral changes in 38.89 % of treated rats and always during the first interval (0 min). Typical writhing-like signs (behavioral category—others) could be fully eliminated with sound or touch during the first interval and spontaneously disappeared before MO, or saline was administered (20 min after 6 mg/kg JMV2959). During the last two intervals of dialysis (160–180 min), we observed increased sleepiness in 6 mg/kg JMV2959 treated rats, which was similar to saline treated rats (Fig. 4f) (JMV2959 6 mg/kg + saline: immobility: $F_{10,50}$ = 11.06 /160–180 min/; no catalepsy; locomotion: $F_{10,50}$ = 9.06 /160–180 min/; no stereotyped activity; others: $F_{10,50}$ = 2.50 /0 min/, in all cases $P < 0.001$; one-way ANOVA RM, Bonferroni's test versus control) (saline + saline: immobility: $F_{10,50}$ = 13.77 /160–180 min/; no catalepsy; locomotion: $F_{10,50}$ = 13.77 /160–180 min/; no stereotyped activity, $P < 0.001$ for all cases; one-way ANOVA RM, Bonferroni's test versus control).

Discussion

Addictive drugs activate dopaminergic (DA) transmission in the nucleus accumbens. Increased extracellular DA levels in

NACSh have been typically associated with the acute rewarding effects of addictive drugs (Koob 1992; Hyman et al. 2006; Koob and Volkow 2010). The observed massive augmentation of extracellular DA concentration in the NACSh following acute administration of MO (5 and 10 mg/kg s.c.) is considered to be associated with MO/opioids reward processing and is supported by the literature (Pothos et al. 1991; Leone et al. 1991; Koob and Volkow 2010). Our results indicate that pretreatment with ghrelin GHS-R1A antagonist (JMV2959) before acute MO injection (5 and 10 mg/kg s.c.) significantly and dose dependently (JMV2959 3 and 6 mg/kg i.p.) reduces MO-induced DA increase in the NACSh, thus implying that the central ghrelin signaling system might be significantly involved in the MO/opioids-induced changes in mesolimbic DA system, associated with reward processing.

The higher dose (6 mg/kg) of JMV2959 did not completely abolish the MO-induced DA effects. One of the possible explanations could be that the ghrelin signaling system plays an important but modulatory role in the initial DA activation of the neural opioid reward system. We have not used higher doses of JMV2959 for the pretreatment, because they themselves produced behavioral changes in rats. Indeed, even the dose 6 mg/kg of JMV2959 produced (in less than 40 % of the rats) temporary behavioral changes, which spontaneously disappeared within 20 min after administration.

As far as we know, the relationship between ghrelin signaling and DA metabolism has not yet been described. As might be expected, the MO-induced accumbens DA increase was associated with a dose-dependent increase of DA metabolism in the nucleus accumbens, with a significant increase of accumbens DOPAC, 3-MT, and HVA, at both MO doses (similar results were reported by Pothos et al. 1991 etc.). JMV2959 itself did not influence accumbens DA concentrations (in agreement with Jerlhag et al. 2009, 2010b). The JMV2959 reduction of MO-induced DA increase was associated with reduction of 3-MT (a product of catechol-O-methyltransferase (COMT)). On the other hand, the JMV2959 reduction of MO-induced DA increase was associated with moderate but significant increase of DOPAC (a product of monoamine oxidase (MAO)) as well as HVA (the final product of DA metabolism). JMV itself (per se) moderately increased levels of DOPAC and HVA, but did not influence that of 3-MT. Thus, it seems that GHS-R1A antagonism might be associated with increased metabolism of DA by MAO. Further investigation is required.

Our behavioral data display the typical biphasic (inhibition-stimulation) effects of MO, which correspond well with literature and our previous studies (Fiserova et al. 1999). MO-induced behavioral changes are thought to reflect activity in the mesostriatal DA system. Behavioral stimulation, involving stereotypical behaviors (confined gnawing, licking, and stereotyped sniffing) is considered to be a sign of nigrostriatal pathway activation (Wise and Bozarth 1987; Koob and Volkow 2010). Pretreatment with JMV2959 significantly and dose dependently reduced (at the higher 6 mg/kg JMV2959 dose also shortened) stereotyped behavior (significantly reduced stereotyped sniffing and gnawing, and abolished licking) at both MO doses, which suggests involvement of ghrelin signaling in some MO-induced behavioral changes, which are considered to be reflecting the DA striatal activation.

In conclusion, our neurochemical and behavioral results indicate a significant involvement of ghrelin signaling in the MO/opiod-induced changes in the mesolimbic DA system, which are associated with neural reward processing. Further investigation into the role of the central ghrelin signaling system in opiod/drug dependence and their treatment is warranted.

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