Neuropeptides 76 (2019) 101937

Contents lists available at ScienceDirect

Neuropeptides

journal homepage: www.elsevier.com/locate/npep

# The additive effect of allopregnanolone on ghrelin's orexigenic effect in rats

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ARTICLE INFO

*Keywords:*
Ghrelin
Allopregnanolone
Brexanolone
Food intake
$GABA_A$-receptor
Patch-clamp
PVN
ARC

ABSTRACT

The progesterone metabolite, allopregnanolone (AlloP), is a $GABA_A$ receptor modulating steroid and is known to have orexigenic and pro-obesity effects. The neurobiological mechanisms underpinning these effects are most likely due to enhanced GABAergic signaling in the lateral arcuate nucleus (ARC) and medial paraventricular nucleus (PVN) of the hypothalamus. Inspired by the finding that GABAergic signaling is also important for the orexigenic effects of the circulating hormone, ghrelin, we sought to determine the extent to which AlloP (one of the most potent endogenous $GABA_A$-receptor modulators) operates alongside ghrelin to enhance food intake. Male rats with *ad libitum* access to standard chow were injected intravenously with AlloP and/or ghrelin, alone or in combination. The intake of the standard chow was greater after AlloP 1 mg/kg together with ghrelin 30 μg/kg than with 30 μg/kg ghrelin alone. Food intake was also increased for the combined treatment of AlloP 0.5 mg/kg + ghrelin 10 μg/kg, AlloP 1 mg/kg + ghrelin 10 μg/kg, and AlloP 0.5 mg/kg + ghrelin 30 μg/kg. There was no significant difference in food intake between the two ghrelin doses or between the two doses of AlloP and the vehicle. In electrophysiological studies, physiologically relevant concentrations of AlloP prolonged the current decay time of spontaneous inhibitory post-synaptic current of dissociated cells of the ARC and PVN. We conclude that AlloP enhances the hyperphagic effect of ghrelin, findings of potential relevance for the hyperphagia associated with the luteal phase of the reproductive cycle.

## 1. Introduction

It is well established that $GABA_A$ receptor modulating steroids (GAMS) have orexigenic and pro-obesity effects, especially progestogens, and the metabolite allopregnanolone (AlloP). During the luteal phase of the menstrual cycle, that can be considered a preparatory phase for a forthcoming pregnancy, energy intake is higher and food cravings are more common (Barr et al., 1995; Cross et al., 2001; Dalvit, 1981; Hormes and Rozin, 2009; Johnson et al., 1994; Reed et al., 2008). This fact underscores the important role of progestogens in ensuring adequate nutrition during pregnancy and lactation (Beksinska et al., 2010; Butte and King, 2005). It appears that some factor formed by the corpus luteum is implicated in these effects since the fluctuation in energy intake is abolished in anovulatory cycles (Ottander et al., 2005). Indeed, some hormonal contraceptives have the side effect of increasing body weight (Bahamondes et al., 2001; Beksinska et al., 2010; Berenson and Rahman, 2009; Bonny et al., 2006; Risser et al., 1999), and the progestogen, medroxyprogesterone-acetate has even been used to improve appetite and hence, the body weight in malnourished patients (Simons et al., 1996). Notably, circulating AlloP levels have been associated with binge eating in women and obesity in both men and women (Menozzi et al., 2002; Monteleone et al., 2003; Predieri et al., 2007). Stress is a known risk factor for obesity (Vieweg et al., 2007), and stress also stimulates the production of AlloP and other $GABA_A$-receptor active steroids in male rats (Purdy et al., 1991) and humans of both sexes (Droogleever Fortuyn et al., 2004).

In rodents, hyperphagia can be induced by the administration of AlloP, and the effect is dose-dependent (Chen et al., 1996; Reddy and Kulkarni, 1998, 1999). Their meal size is increased, which correlates with obesity in rats (Farley et al., 2003; Furnes et al., 2009), and weight gain is also achieved after long term treatment with AlloP (Holmberg et al., 2015). In a choice situation, AlloP does also promote intake of more calorie-dense foods (Holmberg et al., 2014).

Another potent orexigenic substance is ghrelin, which is a stomach-derived peptide hormone (Kojima et al., 1999; Wren et al., 2000) with pro-obesity effects (Tschop et al., 2000). Circulating levels of ghrelin increase pre-prandial, and it has been suggested that ghrelin plays an important role in hunger and meal initiation (Cummings et al., 2001).

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https://doi.org/10.1016/j.npep.2019.101937
Received 15 November 2018; Received in revised form 8 June 2019
Available online 13 June 2019

Ghrelin activates circuits involved in energy homeostasis and appetitive behavior that includes the hypothalamus (Hewson and Dickson, 2000), brainstem (Bailey et al., 2000; Faulconbridge et al., 2008) and mesolimbic reward circuits (Abizaid et al., 2006; Dickson et al., 2010; Egecioglu et al., 2010; Jerlhag et al., 2007; Skibicka et al., 2011; Skibicka et al., 2012). In the arcuate nucleus (ARC), a key target for ghrelin is the orexigenic neuropeptide Y/agouti-related peptide (NPY/AgRP)-expressing cells (Dickson and Luckman, 1997; Qi et al., 2015), which inhibit the anorexigenic pro-opiomelanocortin (POMC) population in the arcuate nucleus (ARC) by a mechanism involving increased GABAergic transmission (Cowley et al., 2003; Gao and Horvath, 2008; Jobst et al., 2004). Indeed, important roles for GABAergic transmission in feeding and ghrelin's orexigenic effects are highlighted by the finding that specific deletion of the vesicular GABA transporter in AgRP-expressing neurons leads to a strongly reduced orexigenic effect of ghrelin (Tong et al., 2008). Both NPY/AgRP- and POMC-expressing neurons project to “second-order neurons” located in, for example, the paraventricular nucleus (PVN), where anorexigenic peptides are released thereby activating catabolic circuits (Gil-Campos et al., 2006; Valassi et al., 2008). Even at this site, inhibitory GABAergic transmission appears to be of importance for food intake (Dos-Santos et al., 2018; Pu et al., 1999). AlloP potentiates the effect of GABA at the $GABA_A$-receptor (Majewska et al., 1986) in a manner similar to that of benzodiazepine, which has a well-documented hyperphagic effect (Cooper, 2005).

Inspired by the important role of GABAergic transmission for the orexigenic effect of ghrelin, we sought to determine what effect AlloP, perhaps the most potent positive endogenous modulator of the $GABA_A$-receptor, would have on ghrelin's orexigenic effects. Additionally, given the large fluctuations in AlloP in normal physiology (Hill et al., 2007; Holzbauer, 1975; Nyberg et al., 2007), together with the large differences in sensitivity thresholds for the $GABA_A$-receptor between different brain areas (Herd et al., 2007), we also investigated the sensitivity of $GABA_A$-receptors to AlloP on cells from the lateral ARC and the medial PVN, two important hypothalamic areas for food intake regulation.

## 2. Materials and methods

### 2.1. Animals

Food intake studies were performed on 24 male Wistar rats (Taconic™, Lille Skensved, Denmark) weighing 150 ± 3.4 g (mean ± st.dev.) upon arrival to the facility. The animals were housed in triads in cages measuring 55 × 35 × 20 cm with *ad libitum* access to food and water. For the first seven days, the animals were acclimatized to the facility and, thereafter, handled on four separate occasions. On two occasions, the rats were habituated to the intravenous (i.v.) injection procedure first by warming the rat's tail in 40–42C° water for 2 min followed by rolling the rat in a blanket and holding it in the injection position. Habituation also included sitting in 40 × 25 × 15 cm cages without bedding for 4 h with *ad libitum* access to food and water. For the electrophysiological studies, 15 male Sprague-Dawley rats (50–150 g from in house breeding) were used. The Ethical Committees for Animal Experimentation at Umeå and Gothenburg, Sweden approved the experimental protocol, and all efforts were made to minimize the number of animals used and their suffering.

#### 2.1.1. Impact of AlloP on ghrelin-induced food intake in rats

The effects of AlloP on ghrelin-induced food intake were determined in adult male rats. The feeding studies were performed during the light phase, when food intake is normally minimal (Holmberg et al., 2013). The rats were injected intravenously with vehicle (10% β-cyclodextrine, Sigma-Aldrich, St Louis, MO, in saline), ghrelin (10 or 30 μg/kg; Tocris Biosciences™, Ellisville, Missouri, USA), AlloP (0.5 or 1 mg/kg, Umecrine AB, Umeå, Sweden), or a combination of ghrelin and AlloP using both of the aforementioned doses of ghrelin and AlloP (in total 1 vehicle and 8 different combinations of doses). The doses of AlloP were selected based on our previous experiments and were well below the threshold for sedation (which begins around 2 mg/kg; unpublished observations). The dose of ghrelin was based on previous reports in which 30 μg/kg ghrelin induced a food intake response close to maximum (Date et al., 2002; Hashimoto et al., 2007). The experiment started 3 h after light onset, a time of the day where the rats are inactive and have a low level of spontaneous eating (Bodosi et al., 2004). Immediately after injection, rats were placed in single cages, and the amounts of ingested food-pellets were measured after 30 min, 1 h, 2 h, 3 h, and 4 h. The experiment was repeated five times with a wash-out period of at least three days. Each rat received a vehicle at one of the five occasions. At the other four occasions it received 4 of the 8 different combinations of doses. The order of treatment and vehicle injections used a balanced design. In total, the vehicle was given to 24 rats, and each of the treatment combinations was given to 12 rats.

#### 2.1.2. Electrophysiological experiments: AlloP's effect on GABAergic transmission in ARC and PVN cells

Here we explored the effects of physiologically relevant concentrations of AlloP on GABAergic transmission, focusing on 2 hypothalamic areas, the lateral ARC and the medial PVN. GABA induces hyperphagia in both these areas which are innervated by ghrelin-responsive cells that release GABA (Cowley et al., 2003; Jobst et al., 2004; Pu et al., 1999; Tong et al., 2008; Valassi et al., 2008). We performed whole-cell patch-clamp recordings from single dissociated cells from these regions. The methods used for electrophysiological recordings and preparation of cells have been described previously (Johansson et al., 1995; Karlsson et al., 1997). In this study, the principal aim was to see how small changes in AlloP concentration affect the response to synaptically-released GABA at the $GABA_A$-receptors. This approach was enabled by patch-clamp recordings from dissociated cells, where AlloP has direct access to its receptor-site (Haage and Johansson, 1999). However, one limitation of the preparation is that we could not study AlloP's effect on ghrelin-evoked GABA transmission. This information would require a more intact preparation with the disadvantages of slow and imprecise exposure to AlloP at the receptor site. The rats were sacrificed by decapitation without the use of any anesthetics. The brain was removed and kept in ice-cold incubation solution. A block of tissue containing the area of interest was dissected. Coronal slices (thickness of 300 μm) were prepared and incubated for at least 1 h in oxygenated incubation solutions at 28 °C. Single cells with adhering synaptic nerve terminals from the lateral part of the lateral ARC and the medial part of the medial PVN were isolated by vibrodissociation (Vorobjev, 1991).

The incubation solution used for preparation and storage of slices contained (in mM) 150 NaCl, 5 KCl, 2.0 $CaCl_2$, 10 HEPES, 10 glucose, and 4.94 Tris-base. The solution was supplemented with $O_2$ gas. The extracellular (control) solution used for recording contained (in mM) 137 NaCl, 5.0 KCl, 1.0 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES, and 10 glucose. The pH was adjusted to 7.4 with NaOH. The intracellular solution for filling the pipettes contained (in mM) 140 K-gluconate, 3.0 NaCl, 1.2 $MgCl_2$, 10 HEPES and 1.0 EGTA pH was adjusted to 7.2 with KOH. Amphoterecin B was dissolved in dimethylsulpoxide and added to a final concentration of 240 μg amphotericin B per ml intracellular solution. Amphotericin-B-perforated patch whole-cell recordings were performed at room temperature (21–23 °C) in the voltage-clamp mode. The perforated patch technique prevents wash-out of cellular components but provides good control over internal concentrations of monovalent ions (Horn and Marty, 1988). In all experiments, after compensation for the liquid-junction potential (Haage et al., 2002), a holding potential of −36 mV was used. The control solution, with or without AlloP, was applied by a gravity-fed fast perfusion system controlled by solenoid valves.

Changes of the graphical pattern detected the spontaneous inhibitory post synaptic currents (sIPSCs). The time-course of decay was

semi-manually fitted to a mono-exponential function ($I = Amplitude_{peak} * e^{-t/\tau}$) by using cursors of the pCLAMP software (Axon Instruments, Foster City, CA, USA) and the time constant ($\tau$) was used for analysis. For further calculation and statistics, Origin (Microcal Software, Northampton, MA, USA) was used. To quantify the effect of AlloP, all $\tau$-values within each cell were converted to ratios based on the mean $\tau$-value under control conditions. For comparisons between different AlloP concentrations within cells from the same area, a one-way ANOVA and post-hoc LSD test was used. For comparisons between the effect of AlloP on cells from medial PVN and lateral ARC, a two-way ANOVA was used.

### 2.2. Statistics

For between-group comparisons in the feeding studies, the one-way ANOVA and post-hoc LSD test was used. Regression analysis was used for AlloP group comparisons; a $p$-value of $< 0.05$ was considered significant.

## 3. Results

### 3.1. Impact of AlloP on ghrelin-induced food intake in rats

Food intake during the first 30 min was significantly increased by AlloP and ghrelin ($F(8,111) = 4,71$; $p = .001$, Fig. 1). The food intake was larger in the presence of 1 mg/kg AlloP together with 30 µg/kg ghrelin than with 30 µg/kg ghrelin alone ($p = .033$) (Fig. 1). A significant increase in food intake relative to vehicle treated animals was also detected for all of the following groups: ghrelin 10 µg/kg ($p = .033$), ghrelin 30 µg/kg ($p = .010$), AlloP 0.5 mg/kg + ghrelin 10 µg/kg ($p = .003$), AlloP 1 mg/kg + ghrelin 10 µg/kg ($p < .001$), AlloP 0.5 mg/kg + ghrelin 30 µg/kg ($p = .002$), AlloP 1 mg/kg + ghrelin 30 µg/kg ($p < .001$). Ghrelin increased food intake at both doses, but AlloP alone did not. There was no significant difference in food intake between the two ghrelin doses nor between the two doses of AlloP. Furthermore, differences were seen between the treatment groups. Increased intake was seen for the AlloP 1 mg/kg + ghrelin 30 µg/kg group compared to ghrelin 10 µg/kg ($p = .011$), ghrelin 30 µg/kg ($p = .033$), AlloP 0.5 mg/kg ($p < .001$), and AlloP 1 mg/kg ($p = .002$). The AlloP 0.5 mg/kg group consumed less than all of the following groups; AlloP 0.5 mg/kg + ghrelin 10 µg/kg ($p = .037$),

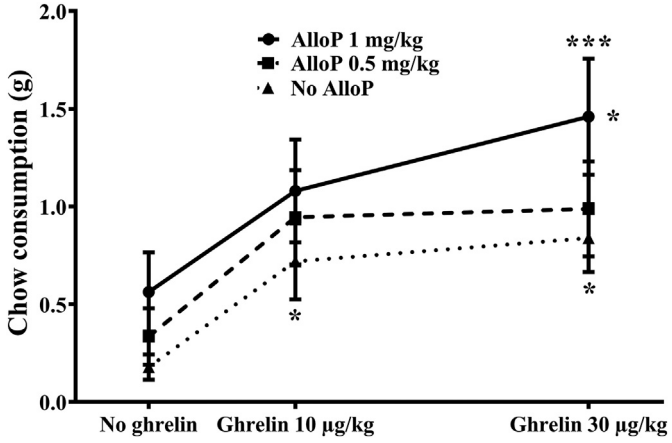


**Fig. 1.** Food intake of chow (g) during the first 30 min after i.v. administration of AlloP and/or ghrelin in non-food-deprived male rats (vehicle group $n = 24$, otherwise $n = 12$). Food intake was greatly increased by AlloP 1 mg/kg in combination with 30 µg/kg ghrelin compared to controls (no ghrelin and no AlloP) (*** = $p < .001$). The addition of AlloP 1 mg/kg was significantly greater than that of 30 µg/kg ghrelin alone (* = $p < .05$). Both doses of ghrelin significantly increased food intake compared to controls (* = $p < .05$). Data presented as mean +/− SEM.

**Table 1**
Food-intake (g) over four hours displayed as mean +/− SEM.

| Groups | | Vehicle | Ghrelin 10 µg/kg | Ghrelin 30 µg/kg | AlloP 0.5 mg/kg | AlloP 1 mg/kg | AlloP 0.5 mg/kg + ghrelin 10 µg/kg | AlloP 1 mg/kg + ghrelin 10 µg/kg | AlloP 0.5 mg/kg + ghrelin 30 µg/kg | AlloP 1 mg/kg + ghrelin 30 µg/kg |
|---|---|---|---|---|---|---|---|---|---|---|
| Hour | 1 | 0.63 +/− 0.11 | 1.08 +/− 0.23 | 1.15 +/− 0.20 | 0.70## +/− 0.18 | 0.99# +/− 0.23 | 0.95# +/− 0.24 | 1.30* +/− 0.27 | 1.14 +/− 0.25 | 1.64*** +/− 0.33 |
| | 2 | 1.19 +/− 0.15 | 1.37 +/− 0.23 | 1.89 +/− 0.28 | 1.07 +/− 0.20 | 1.53 +/− 0.30 | 1.16 +/− 0.30 | 1.60 +/− 0.25 | 1.46 +/− 0.24 | 2.02 +/− 0.33 |
| | 3 | 1.48 +/− 0.13 | 2.16 +/− 0.27 | 1.94 +/− 0.28 | 1.65 +/− 0.23 | 1.71 +/− 0.37 | 1.57 +/− 0.24 | 1.69 +/− 0.26 | 1.98 +/− 0.21 | 2.36 +/− 0.30 |
| | 4 | 2.15 +/− 0.18 | 2.31 +/− 0.28 | 2.50 +/− 0.35 | 2.09 +/− 0.27 | 2.22 +/− 0.40 | 2.38 +/− 0.29 | 1.89 +/− 0.30 | 2.14 +/− 0.22 | 2.54 +/− 0.38 |

Asterisks indicate differences compared to vehicle, * = $p < .05$, *** = $p < .001$.
Hashtags indicate differences compared to the AlloP 1 mg/kg + ghrelin 30 µg/kg group. # = $p < .05$, ## = $p < .01$.

AlloP 0.5 mg/kg + ghrelin 30 μg/kg ($p$ = .026), and AlloP 1 mg/kg + ghrelin 10 μg/kg (p = .011).

At 1 h post injection, food intake was also significantly increased (F(8,111) = 2,21; $p$ = .032) compared to vehicle for the AlloP 1 mg/kg + ghrelin 10 μg/kg group ($p$ = .017), and AlloP 1 mg/kg + ghrelin 30 μg/kg group (p < .001). Increased intake was also noted between the AlloP 1 mg/kg + ghrelin 30 μg/kg group and other treatment groups (Table 1).

At the other time-points after injections (2, 3 and 4 h) no differences were seen between doses and treatments. The transient nature of the effect is consistent with the rapid half-life in plasma after i.v. injections for both AlloP; 44 min (Timby et al., 2006) and ghrelin; 24 min (Vestergaard et al., 2007). To control for individual satiation, the data were divided into two groups based on meal size. It has previously been established that male rats with a similar weight as in the present study, display three meal types depending on ingestion rate (Demaria-Pesce and Nicolaidis, 1998). A scatter-plot of the food intake during the first 30 min revealed two distinct groups: one group that ate very little (in the interval around 0 to 0.5 g) and another in which a clear feeding response could be detected (in the interval around 0.9 g and over). Therefore, we divide the data into two groups: one group containing low-eaters (ranging from 0 to 0.9 g of chow) and another group containing animals that ate at least the amount of food considered to be the size of a single meal (0.9 g and over, $n$ = 48) (Fig. 2). This division enabled us to differentiate between animals that were sufficiently motivated to eat and animals that were not motivated. Even with this criterion, the effect remained. Also, in the more motivated group that ate > 0.9 g, AlloP added to the hyperphagic effect of ghrelin (F(8,39) = 2,29; $p$ = .41). The AlloP 1 mg/kg + ghrelin 30 μg/kg consumed more than the vehicle group ($p$ = .017), and also when compared to the ghrelin 10 μg/kg ($p$ = .005), ghrelin 30 μg/kg ($p$ = .002), and the AlloP 1 mg/kg group ($p$ = .045). In addition the AlloP 1 mg/kg + ghrelin 10 μg/kg consumed more than the ghrelin 30 μg/kg group ($p$ = .032) (Fig. 3).

The data can also be expressed as food intake (g) per body weight (kg). This recalculation produced similar results as the food intake presented in grams. Differences were seen after the first 30 min (F(8,111) = 4,80; $p$ < .001), and after 1 h (F(8,111) = 2,41; $p$ = .019).

### 3.2. Impact of AlloP on GABAergic transmission in lateral ARC and medial PVN cells

Both the lateral ARC and medial PVN are innervated by GABAergic synapses that originate from ghrelin-responsive cells and have an essential role for the regulation of food intake (Cowley et al., 2003; Jobst et al., 2004; Pu et al., 1999; Tong et al., 2008; Valassi et al., 2008). The sIPSCs were recorded from 30 cells from the medial PVN in the voltage-clamp mode at a holding potential of −36 mV (Fig. 4A–C). Under control conditions, the time-course of decay (τ) of the recorded sIPSCs fitted well to a mono-exponential function and were normally distributed ($n$ = 1846 events) with a mean and SEM of 25.3 ± 0.3 ms. All of these properties suggest that the currents originate from the presynaptic release of GABA which has previously been described using this preparation on cells from the medial preoptic nucleus (Haage et al., 2002; Haage et al., 1998). AlloP concentration-dependently prolonged τ which already at 2 nM was 1.22 ± 0.03 times longer (mean and S.E.M.) compared to control (based on 522 events pooled from 16 cells p < .001). The effect on τ showed a linear relationship to the concentration of AlloP in the investigated range (Fig. 4B). At 20 nM AlloP, τ was 3.33 ± 0.1 times longer than control (mean ± SEM, based on 759 events pooled from 28 cells). The values for the other concentrations are based on: for 4 nM; 378 events from 9 cells, for 6 nM; 206 events from 4 cells, for 10 nM; 407 events from 9 cells. We also performed some complementary recordings in a few cells ($n$ = 7) from the lateral ARC. Also, here the current decay time of sIPSCs were normally distributed ($n$ = 431 events) with an average and SEM of 19.5 ± 0.4 ms, and we concluded that the effect of AlloP at these cells was almost identical to that at medial PVN cells (Fig. 4B). For the recorded cells from the lateral ARC the values for the different concentrations are calculated based on: for 2 nM; 27 events from 2 cells, for 10 nM; 85 events from 7 cells, for 20 nM; 100 events from 7 cells.

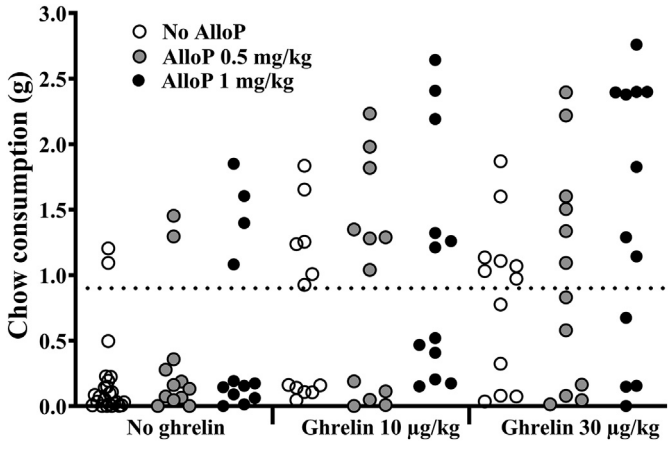


**Fig. 2.** Scatter plot over food intake of chow (g) during the first 30 min after i.v. administration of AlloP and/or ghrelin in non-food-deprived male rats (vehicle group n = 24, otherwise n = 12). The abscissa at 0.9 g indicates two populations of committed and non-committed rats.

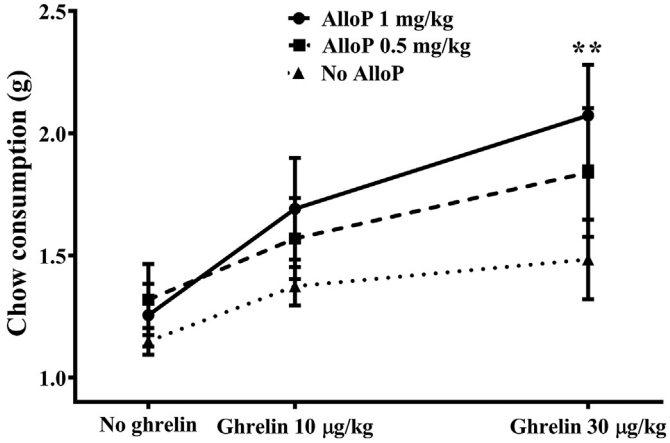


**Fig. 3.** Food intake of normal chow (g) during the first 30 min after i.v. administration of AlloP and/or ghrelin in animals that ate > 0.9 g. AlloP (1 mg/kg) combined with ghrelin (30 μg/kg) increased chow intake compared to ghrelin 30 μg/kg alone (** = $p$ < .01). Data presented as mean +/− SEM.

## 4. Discussion

Here we demonstrate that AlloP enhances the orexigenic effects of ghrelin. This finding implies that ghrelin and AlloP exert their orexigenic effects, at least in part, by different mechanisms. This effect remained and was even more pronounced when the animal's individual satiety and hunger drive was considered. Ghrelin increased food intake which is in compliance with its established effect on meal initiation (Cummings, 2006; Cummings et al., 2001) and motivated behavior for food (Egecioglu et al., 2010; Perello et al., 2010; Skibicka et al., 2011; Skibicka et al., 2012). A common denominator for the known neurobiological effects of ghrelin and AlloP is the GABAergic signaling system. Furthermore, AlloP is one of the most potent endogenous positive modulators of the $GABA_A$-receptor (Majewska et al., 1986) and has an important role in GABAergic signaling in food intake initiation. It has also been shown by local application of the $GABA_A$-receptor

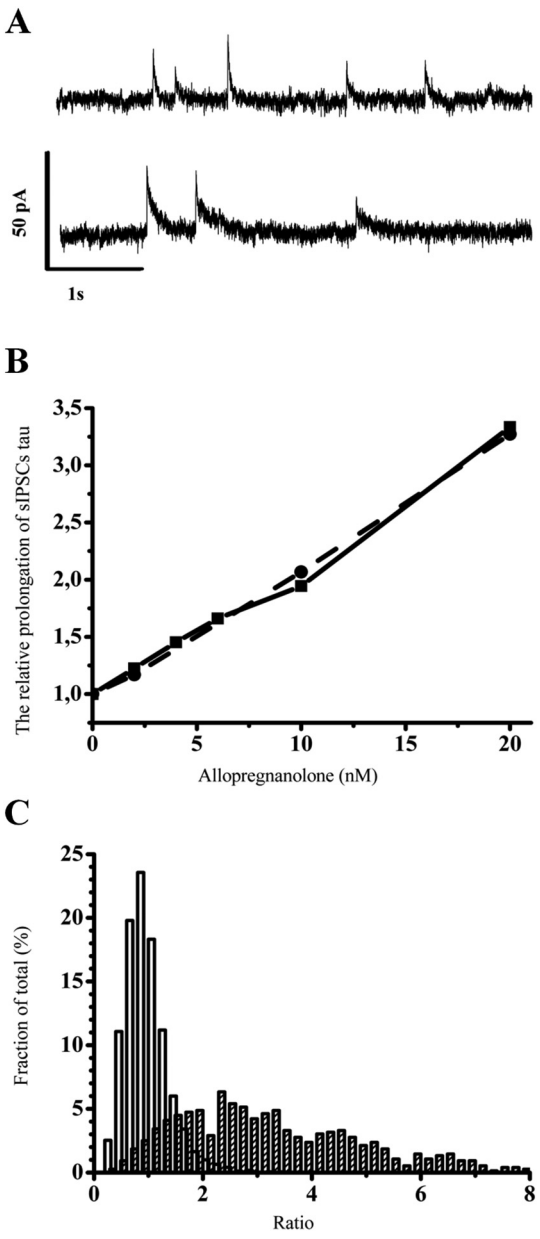


**Fig. 4.** AlloP prolonged the sIPSCs in cells from the PVN and ARC cells. A: A recording of sIPSCs from a medial PVN cell under control conditions (top fig) and in the presence of 20 nM AlloP (bottom fig). B: The relative prolongation of sIPSCs τ in the presence of AlloP compared to control. Solid lines; sIPSCs from medial PVN. Dashed line; sIPSCs from lateral ARC. C: Normalized distribution of events in 20 nM AlloP (dashed bars) and their corresponding values in controls (white bars) (bin width = 0.2).

agonist muscimol in several hypothalamic and extra hypothalamic sites (Arnt and Scheel-Kruger, 1979; Przewlocka et al., 1979; Pu et al., 1999; Tsujii and Bray, 1991). There may also be some interplay between the two substances since ghrelin requires an intact transmission of GABA to function (Tong et al., 2008; Wu et al., 2009). Interestingly, ghrelin may regulate the effect of AlloP is supported by that two of three previous studies reporting a hyperphagic effect of AlloP involved studies in food-deprived animals (Chen et al., 1996; Reddy and Kulkarni, 1998, 1999), a condition where ghrelin levels are high (Tschop et al., 2000). Other studies could not detect any effect of AlloP on food-intake in non-deprived animals (Fudge et al., 2006; Higgs and Cooper, 1998).

Based on the knowledge about the function of AlloP and ghrelin, potential hypothalamic sites at which AlloP may affect feeding-regulating circuits include the ARC (especially more lateral areas where POMC-expressing neurons are located) and the medial PVN (Cowley et al., 2003; Jobst et al., 2004). To explore these potential sites/mechanisms for AlloP's effects, we performed several electrophysiological studies in dissociated cells taken from these brain areas. Notably, the sensitivity of the $GABA_A$-receptor to AlloP can vary substantially between subunit compositions as well as brain areas (Belelli et al., 2002; Herd et al., 2007). Therefore it is important to investigate what effect AlloP has on GABA-mediated currents at neurons exactly in the PVN and the ARC. Even if we cannot be sure how many of the recorded cells that contained GABAergic synapses originating from just ghrelin-responsive cells we could at least conclude that AlloP in the low nanomolar range, which correlates to physiological levels (Nyberg et al., 2007), prolongs the sIPSCs at cells within both medial PVN and lateral ARC. Since a specific loss of GABA transmission from AgRP-neurons located in the medial part of the ARC dramatically reduced the hyperphagic effect of ghrelin (Tong et al., 2008; Wu et al., 2009) it is expected that an amplification of the GABAergic transmission in this area (*i.e.*, by AlloP) would increase hyperphagia. It should be taken into consideration, however, that enhanced GABAergic transmission in another hypothalamic area, the lateral hypothalamus, has been reported to reduce food intake (Tsujii and Bray, 1991), although it is not known if AlloP enhances signaling at this site. One possible mechanism for the AlloP induced increase in food intake is that AlloP acts downstream of the ghrelin sensitive GABAergic neurons involved in feeding regulation by potentiating the postsynaptic response to GABA (Holmberg et al., 2018).

In conclusion; the results demonstrate an additive effect of AlloP on the orexigenic effect of ghrelin and appear to involve GABAergic signaling in key brain areas involved in feeding, including ghrelin-responsive orexigenic circuits. Therefore, it may be suggested that AlloP is a physiologically relevant signal for enhancing total food intake, a known determinant for over-nutrition and obesity.

## Acknowledgment

Dr. David Haage for excellent assistance with conducting the experiments, and for great overall support.

The research leading to these results has received funding from the European Community's Seventh Framework Programme (FP7/2007-2013 under grant agreement no 245009 and FP7-HEALTH-2009-241592), the Swedish Medical Research Council (2006-5663, 2009-5266, 4x-11198), Novo Nordisk Fonden (GeA/AIR), ALF Göteborg (SU7601) and the Swedish Foundation for Strategic Research of the Sahlgrenska Center for Cardiovascular and Metabolic Research (A305-188). Umeå University Foundations, Västerbotten läns landsting, and Umeå Biotech Incubator.

## Declaration of interest

Professor Torbjörn Bäckström is a shareholder and board member of Umecrine AB. None of the other authors have any competing interest.

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