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ORIGINAL ARTICLE

# Activation of nucleus accumbens μ-opioid receptors enhances the response to a glycaemic challenge

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## Abstract

Opioids are known to affect blood glucose levels but their exact role in the physiological control of glucose metabolism remains unclear. Although there are numerous studies investigating the peripheral effects of opioid stimulation, little is known about how central opioids control blood glucose and which brain areas are involved. One brain area possibly involved is the nucleus accumbens because, as well as being a key site for opioid effects on food intake, it has also been implicated in the control of blood glucose levels. Within the nucleus accumbens, μ-opioid receptors are most abundantly expressed. Therefore, in the present study, we investigated the role of μ-opioid receptors in the nucleus accumbens in the control of glucose metabolism. We show that infusion of the μ-opioid receptor agonist [D-Ala$^2$, *N*-MePhe$^4$, Gly-ol]-enkephalin (DAMGO) in the nucleus accumbens by itself does not affect blood glucose levels, but it enhances the glycaemic response after both an insulin tolerance test, as well as a glucose tolerance test. These findings indicate that the nucleus accumbens plays a role in the central effects of opioids on glucose metabolism, and highlight the possibility of nucleus accumbens μ-opioid receptors as a therapeutic target for enhancing the counter-regulatory response.

KEYWORDS

DAMGO, glucose metabolism, insulin tolerance test, nucleus accumbens, μ-opioid receptor

## 1 | INTRODUCTION

Opioids, both synthetic and endogenous, can modulate blood glucose levels [1-7]. Although the first reports of morphine-inducing hyperglycaemia date back to the 1920s [7], the exact role of endogenous opioids in the physiological control of glycaemia still remains to be elucidated. For example, both increases and decreases in glycaemia upon i.v. infusion of the endogenous opioid β-endorphin have been found [4,5,8]. Furthermore, mice lacking the μ-opioid receptor (which binds opioids such as morphine and β-endorphin) not only show improved glucose tolerance [9], but also develop insulin resistance more rapidly than wild-type mice [10]. These discrepancies could be explained by the fact that multiple factors, including body weight (BW) [11], route of administration [2,3] and blood glucose levels itself,[5,11] can alter the effects opioids have on glucose metabolism.

To unravel the physiological role of opioids in glucose control, a better understanding of particularly the central effects of opioids on glycaemia is needed. The brain is an important site for the production of endogenous opioids [12]. Furthermore, the central infusion of opioids, in amounts that are insufficient to induce changes when infused peripherally, alters glycaemia [2,13]. To investigate central opioid stimulation and glucose control, i.c.v. infusion of opioids has been used [2,13,14], thereby simultaneously affecting multiple brain areas. Because opioid receptors are expressed throughout the brain [15], it is unclear which brain areas are responsible for the changes seen in glycaemia upon i.c.v. infusion of opioids.

One area of particular interest is the nucleus accumbens (NAC). We have shown that the NAC has a glucoregulatory function because glycaemia was affected by altering NAC activity via deep brain stimulation, direct activation of dopamine-receptor 1 expressing NAC neurones or increasing NAC extracellular serotonin concentrations [16-18]. The NAC is also an important area for the effects of opioid transmission. It contains several types of opioid receptors, with the μ-opioid receptor showing the strongest expression [15] and comprising a key site for the effects of opioids on food intake [19]. We therefore hypothesise that the central effects of opioids on glucose metabolism are mediated by the NAC. To test this hypothesis, we targeted the most highly expressed opioid receptor in the NAC, the μ-opioid receptor [15], using intra-NAC infusion of the μ-opioid receptor specific agonist [D-Ala$^2$, *N*-MePhe$^4$, Gly-ol]-enkephalin (DAMGO) and assessed the effects of NAC μ-opioid receptor activation on basal glycaemia, insulin tolerance and glucose tolerance.

## 2 | MATERIALS AND METHODS

### 2.1 | Animals

Male Wistar rats (Charles River Breeding Laboratories, Sulzfeld, Germany) weighing 240–280 g at arrival were used in the animal facility of The Netherlands Institute for Neuroscience (NIN; Amsterdam, The Netherlands). Rats were housed under a 12:12 h light/dark photocycle (lights on 7.00 AM) at 21 ± 2˚C) and 60% ± 5% relative humidity rooms with background noise (radio) during the entire experiment. During a 1 week acclimatisation, rats were group-housed and had free access to a container with nuggets of a nutritionally-complete high-carbohydrate control diet (chow; Teklad Global Diet 2918; 24% protein, 58% carbohydrate and 18% fat; 3.1 kcal g$^{-1}$; Envigo, Horst, The Netherlands) and a bottle of tap water. All procedures were approved by the Animal Ethics Committee of the Royal Dutch Academy of Arts and Sciences (KNAW, Amsterdam, The Netherlands) and were performed in accordance with the guidelines on animal experimentation of The Netherlands Institute for Neuroscience.

### 2.2 | Surgeries

Rats were anaesthetised with an i.p. injection of a mixture of 80 mg kg$^{-1}$ ktamine (Eurovet Animal Health, Bladel, The Netherlands), 8 mg kg$^{-1}$ Rompun® (xylazine; Bayer Health Care, Mijdrecht, The Netherlands) and 0.1 mg kg$^{-1}$ atropine (Pharmachemie B.V., Haarlem, The Netherlands). A silicone catheter was implanted in the jugular vein, according as described previously [20], for i.v. infusion of insulin or glucose, as well as for blood sampling. After catheter implantations, rats were fixed in a stereotact (Kopf®; David Kopf instruments, Tujunga, CA, USA) and two 26-gauge stainless steel guide cannulas (C315G-SPC 8 mm; Plastics One, Bilaney Consultants GmbH, Düsseldorf, Germany) aimed bilaterally at the NAC were implanted with a 10° angle (anteroposterior +1.4 mm, mediolateral ±2.8 mm, dorsoventral −7.1 mm). Catheters and cannulas were fixed on the skull with dental cement and anchor screws. Rats received Carprofen (5 mg kg$^{-1}$ BW, s.c.) during surgery and the first post-surgery day. After surgery, rats were housed individually. During the recovery period of 14 days, food, water intake and BW were measured daily. Jugular vein catheters were flushed twice a week.

### 2.3 | Experimental procedures

After the recovery period and 1 week before the experiment itself, all rats received a bilateral NAC infusion with vehicle (0.3 μL 0.9% phosphate-buffered saline [PBS]; Fresenius Kabi GmbH, Zeist, The Netherlands) to habituate all rats to the handling and NAC infusion procedure.

The evening before the experiment, rats were connected to a multichannel fluid infusion swivel (Instech Laboratories, Plymouth Meeting, PA, USA) to adapt. Food was restricted to 20 g of chow to avoid differences in the nutritional state and basal blood glucose concentrations of the rats. On the day of the experiment, left-over food was removed at 8.00 AM and rats were connected to the blood-sampling catheter. Five minutes before intra-NAC infusion, a baseline blood sample was collected. At mid-day, rats received a bilateral intra-NAC infusion of either vehicle (0.3 μL 0,9% PBS;

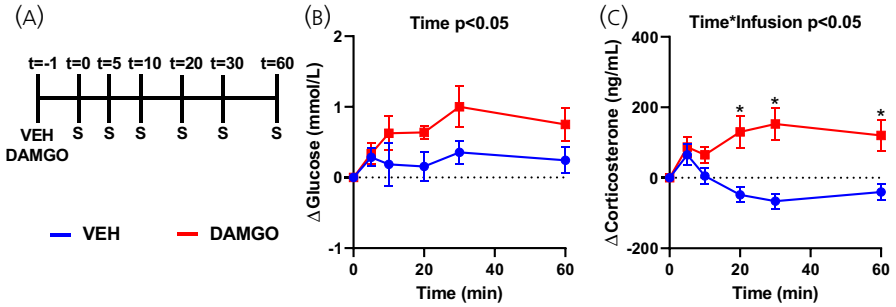


**FIGURE 1** [D-Ala2, *N*-MePhe4, Gly-ol]-enkephalin (DAMGO) infusion does not alter basal glycaemia. A, Experimental outline, with time (min). VEH, vehicle; S, blood sample taken. B, Glucose measured in blood, relative to $t = 0$. C, Corticosterone measured in plasma, relative to $t = 0$. For statistical outcomes, see Table 1. Data are shown as the mean ± SEM. $^{*}p < .05$

Fresenius Kabi GmbH) or DAMGO (0.25 μg in 0.3 μL of 0.9% PBS; Sigma-Aldrich, St Louis, MO, USA). Injectors were left in the guide cannula for 1 min after completion of the infusion to allow for diffusion. During intra-NAC infusion, rats were removed from cages and received infusion while being handled, whereas insulin/glucose injection and blood sampling (200 μL per sample, an equivalent amount of saline was administered after each sample) occurred inside their home cages when the rats were freely moving.

For the insulin tolerance test (ITT), rats received an intra-NAC infusion (VEH, $n = 10$; DAMGO, $n = 8$), and straight after a blood sample for $t = 0$ was collected and insulin bolus was injected i.v. (0.1 IU $kg^{-1}$ BW; Actrapid; Novo Nordisk, Bagsværd, Denmark). For the basal glycaemia measurements, the intra-NAC infusion (VEH, $n = 5$; DAMGO, $n = 8$) occurred at mid-day and blood sampling started directly after. To test whether DAMGO needed 20 min to exert its effects, in the same rats, 7 days after testing for effects on basal glycaemia, an ITT was performed where vehicle or DAMGO was infused 20 min prior to insulin bolus. Rats received intra-NAC infusion (VEH, $n = 6$; DAMGO, $n = 5$) at mid-day and, at 12.20 PM, a blood sample was collected for $t = 0$ and insulin bolus was injected. Rats receiving DAMGO during the basal glycaemia measurements received vehicle in the ITT. For the glucose tolerance test (GTT), right after intra-NAC infusion (VEH, $n = 8$; DAMGO, $n = 8$), a blood sample for $t = 0$ was collected and a glucose bolus was infused i.v. (500 mg $kg^{-1}$ BW; Sigma-Aldrich). At the end of the experiment ($t = 60$), animals were anaesthetised with a $CO_2/O_2$ mixture (6:4) and killed by decapitation. Brains and part of the liver were then rapidly removed, frozen on dry ice and stored at −80°C.

## 2.4 | Glycaemia and hormone measurement

Blood glucose concentrations were measured directly during the experiment, using a glucose monitor device (Freestyle Freedom Lite; Abbott, Hoofddorp, The Netherlands). Blood samples were immediately chilled on ice and centrifuged (4000 *g* for 15 min). Plasma samples were stored at −20°C until further analysis. Plasma concentrations of insulin, glucagon and corticosterone were measured *in duplo* using radioimmunoassay kits (Millipore, St Charles, MO, USA, and MP Biochemicals, Costa Mesa, CA, USA, respectively).

## 2.5 | Cannula placement

Brain tissue sections were cut at 35 μm and mounted on Superfrost Plus slides (Fisher, Gerhard Menzel GmbH, Germany), fixed with a 4% paraformaldehyde solution and stained for Nissl staining with thionine. Stained sections were examined under the microscope to determine the placement of the cannula (see Supporting information, Figure S1), and animals with unilateral or bilateral misplacement were excluded from the analysis.

## 2.6 | Glycogen measurement

A small piece (approximately 10 mg) was dissected from frozen livers, 200 μL of Milli=Q (Merck Millipore, Burlington, MA, USA) was added and tissue was homogenised using an ULTRA THURRAX homogeniser (IKA, Staufen, Germany). Glycogen was measured using an Glycogen Assay kit (Abcam, Cambridge, UK). Glycogen concentrations were normalised by total protein concentration measured in liver homogenates using BioRad Protein Assay for spectrophotometry (Bio-Rad, Hercules, CA, USA).

## 2.7 | Statistical analysis

Data are shown as the mean ± SEM. For all experiments, except when intra-NAc infusion occurred 20 min before an ITT, data are shown relative to baseline (as measured at $t = 0$). For effects on glycaemia and hormone concentrations, a repeated-measure analysis of variance (rmANOVA) was performed using Prism (GraphPad Software Inc., San Diego, CA, USA) and, when appropriate, a post-hoc Fisher's least significant difference test was used to compare individual time points. A Student's *t* test was performed to compare glycogen concentrations. $p < .05$ was considered statistically significant.

# 3 | RESULTS

## 3.1 | DAMGO infusion into the NAC does not affect basal glycaemia

We first investigated whether bilateral infusion of DAMGO into the NAC affected basal glycaemia (Figure 1A). We observed a main

**TABLE 1** rmANOVA outcomes

| | | Time effect | | Infusion effect | | Time × Infusion effect | |
|---|---|---|---|---|---|---|---|
| Basal glycaemia | Glucose | **$p = .0018$** | $F_{5,65} = 4.443$ | $p = .1863$ | $F_{1,13} = 3.435$ | $p = .2800$ | $F_{5,65} = 1.524$ |
| | Corticosterone | $p = .1685$ | $F_{5,65} = 1.899$ | **$p = .0122$** | $F_{1,13} = 14.59$ | **$p = .0014$** | $F_{5,65} = 6.687$ |
| ITT | Glucose | **$p < .0001$** | $F_{5,80} = 47.21$ | $p = .2259$ | $F_{1,16} = 1.586$ | **$p = .0014$** | $F_{5,80} = 4.385$ |
| | Glucagon | **$p < .0001$** | $F_{5,80} = 9.430$ | $p = .5525$ | $F_{1,16} = 0.3682$ | **$p = .0136$** | $F_{5,80} = 3.078$ |
| | Corticosterone | $p = .0880$ | $F_{5,85} = 1.992$ | **$p = .0062$** | $F_{1,17} = 9.727$ | **$p < .0001$** | $F_{5,85} = 6.961$ |
| ITT −20 | Glucose | **$p < .0001$** | $F_{5,45} = 66.48$ | $p = .1010$ | $F_{1,9} = 3.336$ | $p = .3022$ | $F_{5,45} = 1.250$ |
| | Glucagon | **$p < .0001$** | $F_{5,45} = 8.262$ | $p = .5322$ | $F_{1,9} = 0.4219$ | $p = .4589$ | $F_{5,45} = 0.9491$ |
| | Corticosterone | $p = .2394$ | $F_{5,45} = 1.409$ | $p = .1252$ | $F_{1,9} = 2.857$ | $p = .2365$ | $F_{5,45} = 1.417$ |
| GTT | Glucose | **$p < .0001$** | $F_{5,80} = 205.0$ | **$p = .0417$** | $F_{1,16} = 6.977$ | $p = .1932$ | $F_{5,80} = 1.604$ |
| | Insulin | **$p < .0001$** | $F_{5,75} = 160.5$ | $p = .3548$ | $F_{1,15} = 0.7168$ | $p = .1747$ | $F_{5,75} = 1.345$ |
| | Corticosterone | **$p < .0001$** | $F_{5,80} = 8.420$ | $p = .1351$ | $F_{1,16} = 1.196$ | **$p < .0001$** | $F_{5,80} = 15.17$ |

Abbreviations: GTT, glucose tolerance test; ITT −20, insulin tolerance test where [D-Ala2, *N*-MePhe4, Gly-ol]-enkephalin (DAMGO) was administered 20 min prior to the insulin tolerance test; ITT, insulin tolerance test.

effect of Time (Table 1), but no Infusion or Time × Infusion interacting effect, indicating that DAMGO infusion did not alter basal glycaemic levels (Figure 1B). The DAMGO infusion did increase plasma corticosterone levels (Time × Infusion, $p < .0001$), leading to significantly higher concentrations in DAMGO-infused rats compared to vehicle-infused rats from 20 min after infusion onwards (Figure 1C).

## 3.2 | Intra-NAC DAMGO infusion enhances the glycaemic response after an i.v. insulin injection

To test the involvement of NAC μ-opioid receptors in insulin sensitivity, we infused DAMGO in the NAC, right before an i.v. ITT (Figure 2A). A significant Time × Infusion interaction ($p = .0014$) was found for glycaemia (Table 1), indicating that the intra-NAC DAMGO infusion altered the response to an insulin bolus. Post-hoc testing revealed that DAMGO prolongs the insulin-induced drop in glycaemia because DAMGO-treated rats had lower glycaemic values at $t = 10$ min (Figure 2B). These changes are possibly due to a larger decrease in glucagon secretion (Time × Infusion, $p = .0136$) because glucagon concentrations were significantly lower in DAMGO-treated animals at $t = 5$ (Figure 2D). Moreover, at $t = 30$–$60$ min, we observed higher glycaemic values in the DAMGO-treated animals, indicating an enhanced counter-regulatory response (Figure 2B). Again, DAMGO infusion in the NAC increased plasma corticosterone levels (Time × Infusion, $p < .0001$), specifically $t = 30$–$60$ min (Figure 2C). To test whether the increased glycaemia is a result of glycogen breakdown, we measured glycogen content in liver samples. No differences were seen between vehicle or DAMGO-treated animals, indicating

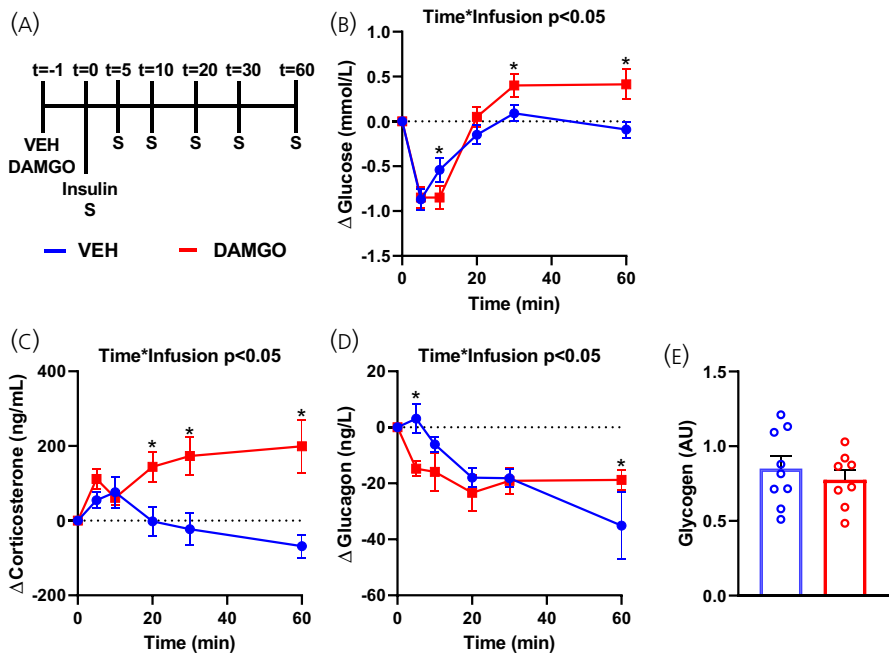


**FIGURE 2** [D-Ala2, *N*-MePhe4, Gly-ol]-enkephalin (DAMGO) infusion enhances the glycaemic response after an i.v. insulin infusion. A, Experimental outline, with time (min). VEH, vehicle; S, blood sample taken. B, Glucose measured in blood, relative to $t = 0$. C, Corticosterone measured in plasma, relative to $t = 0$. D, Glucagon measured in plasma, relative to $t = 0$. E, Glycogen content in liver standardised to total protein. For statistical outcomes, see Table 1. Data are shown as the mean ± SEM. *$p < .05$

that glycogen breakdown is not the main source of the increased glycaemic levels seen in DAMGO treated animals (Figure 2E).

Because we primarily find effects of DAMGO 20 min after infusion, we investigated whether we missed an effect on the earlier stage of the response ($t = 0$–20 min) as a result of DAMGO needing 20 min to exert its effects. We therefore repeated the ITT, but infused DAMGO earlier (i.e., 20 min before the i.v. insulin injection) (Figure 3A). Because DAMGO infusion affected glucagon and corticosterone levels at $t = 0$ (Table 2), we report absolute levels of glucose and hormones and do not express any measures relative to $t = 0$ min. DAMGO infusion 20 min prior to insulin infusion had no effect on glycaemia (Figure 3B and Table 1), plasma corticosterone levels (Figure 3C and Table 1), plasma glucagon levels (Figure 3D and Table 1) or liver glycogen content (Figure 3E).

### 3.3 | Intra-NAC DAMGO infusion also affects the glycaemic response after i.v. glucose injection

Lastly, we tested whether intra-NAC infusion of DAMGO also affected glucose tolerance. When DAMGO was administered prior to an i.v. GTT, we found significant effects of Infusion ($p = .0178$) and Time ($p < .0001$) on glycaemia, but no significant Infusion or Interaction effects of DAMGO on insulin secretion were observed (Figure 4B, C and Table 1). Post-hoc testing revealed no differences in the initial peak in glycaemia (Figure 4B), but, again, glycaemic levels were significantly increased during the counter-regulatory phase (20–60 min after the glucose injection) (Figure 4B). rmANOVA also showed a significant Time × Infusion effect ($p < .0001$) of DAMGO on corticosterone release (Table 1) as a result of initially lower levels

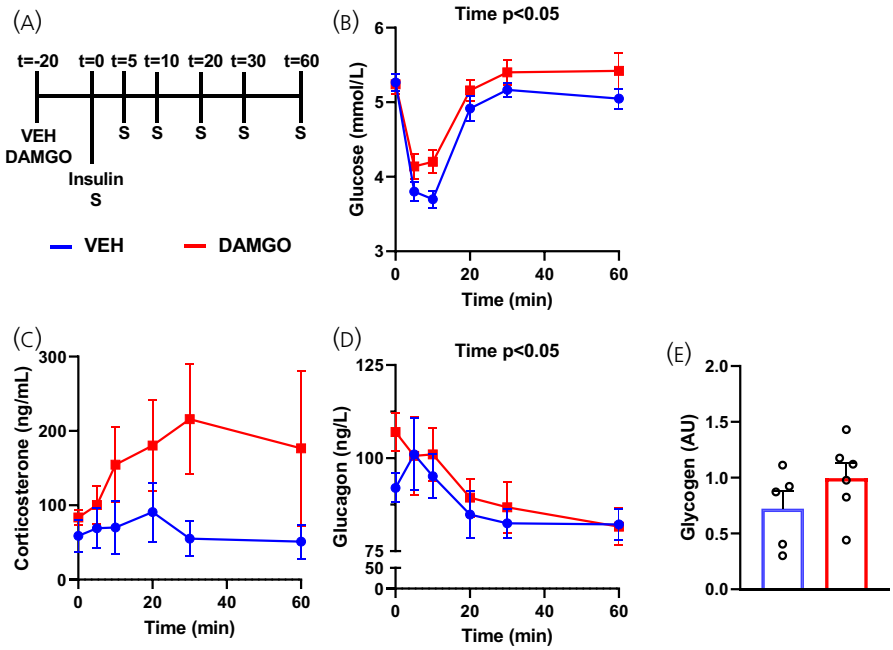


**FIGURE 3** [D-Ala2, *N*-MePhe4, Gly-ol]-enkephalin (DAMGO) infusion 20 min prior to insulin infusion, does not alter the glycaemic response after an i.v. insulin infusion. A, Experimental outline, with time (min). VEH, vehicle; S, blood sample taken. B, Glucose measured in blood. C, Corticosterone measured in plasma D, Glucagon measured in plasma. E, Glycogen content in liver standardised to total protein. For statistical outcomes, see Table 1. Data are shown as the mean ± SEM. *$p < .05$

**TABLE 2** Plasma levels of glucose and hormones at $t = 0$, prior to administration of insulin or glucose

| | | Saline | DAMGO | *p* value |
|---|---|---|---|---|
| Basal glycaemia | Glucose | 4.82 ± 0.29 | 4.84 ± 0.17 | 0.956 |
| | Corticosterone | 70.88 ± 30.81 | 87.89 ± 36.01 | 0.750 |
| ITT | Glucose | 4.82 ± 0.07 | 4.74 ± 0.13 | 0.584 |
| | Glucagon | 112,50 ± 5,78 | 118,13 ± 7,58 | 0,565 |
| | Corticosterone | 94,25 ± 33,45 | 44.14 ± 8.87 | 0.151 |
| ITT −20 | Glucose | 5.20 ± 0.31 | 5.24 ± 0.30 | 0.826 |
| | Glucagon | 90.57 ± 9.48 | 107.00 ± 11.55 | **0.026** |
| | Corticosterone | 55.36 ± 48.75 | 83.50 ± 21.22 | 0.258 |
| GTT | Glucose | 5.15 ± 0.12 | 5.04 ± 0.08 | 0.451 |
| | Insulin | 1.59 ± 0.32 | 1.55 ± 0.25 | 0.920 |
| | Corticosterone | 100.63 ± 36.32 | 96.72 ± 36.52 | 0.941 |

*Note:* Data are shown as the mean ± SEM.

Abbreviations: GTT, glucose tolerance test; ITT −20, insulin tolerance test where [D-Ala2, *N*-MePhe4, Gly-ol]-enkephalin (DAMGO) was administered 20 min prior to the insulin tolerance test; ITT, insulin tolerance test.

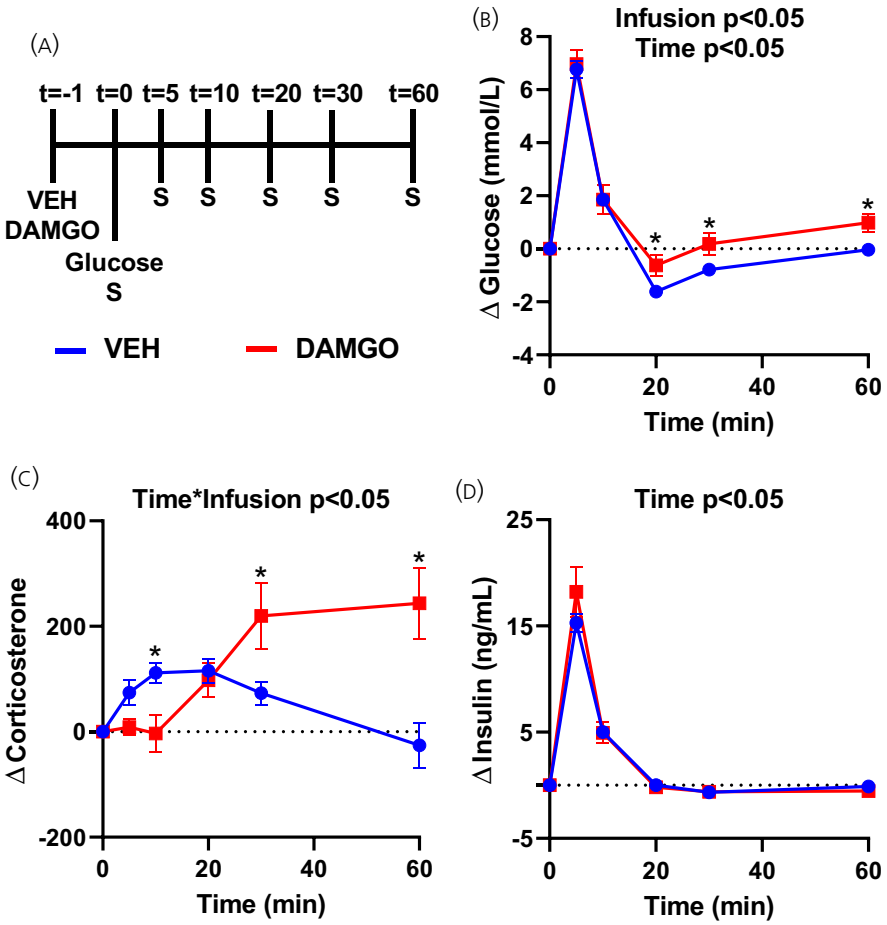


**FIGURE 4** [D-Ala2, *N*-MePhe4, Gly-ol]-enkephalin (DAMGO) infusion enhances the glycaemic response after an i.v. glucose infusion. A, Experimental outline, with time (min). VEH, vehicle; S, blood sample taken. B, Glucose measured in blood, relative to $t = 0$. C, Corticosterone measured in plasma, relative to $t = 0$. D, Insulin measured in plasma, relative to $t = 0$. For statistical outcomes, see Table 1. Data are shown as the mean ± SEM. $^*p < .05$

of corticosterone at $t = 10$, followed by significantly higher plasma corticosterone concentrations from $t = 30$–60 min in DAMGO-treated compared to vehicle-treated animals (Figure 4D).

## 4 | DISCUSSION

In the present study, we found that activation of NAC μ-opioid receptors enhanced the glycaemic response after an insulin or glucose tolerance test, whereas it did not affect basal glycaemia. These results indicate a role for the NAC in the central control of opioids on glucose metabolism.

Previous studies have investigated the effects of i.c.v. infusion of the endogenous opioid β-endorphin [13], the synthetic μ-opioid receptor agonist morphine [3] or DAMGO [14] on blood glucose levels. In line with our findings, all three studies reported an increase in glycaemia upon opioid stimulation [3,13,14]. Unlike our findings, i.c.v. infusion of morphine and β-endorphin caused hyperglycaemia in the absence of any glycaemic challenge [3,13]. Because i.c.v. infusions reach many multiple brains areas, it appears that, although NAC μ-opioid receptor activation specifically affects the counter-regulatory response, activation of μ-opioids receptors in other brain areas may increase glycaemia even in the absence of a glycaemic challenge. Alternatively, this difference could be a result of the nature of the agonists used. Although β-endorphins, morphine and DAMGO all bind to the μ-opioid receptor, they have different affinities and potencies [21,22]. Intracerebroventricular infusion of DAMGO similarly increased glycaemia in response to an ITT or GTT [14], underlining the role of the μ-opioid receptor in the control of the response to a glycaemic challenge. However, i.c.v. infusion of DAMGO also altered insulin release in response to a glucose injection, which we did not observe after intra-NAC DAMGO infusion. Again, this effect could be mediated by other μ-opioid receptor expressing brain areas, such as the hypothalamus [23]. Overall, we conclude that the NAC plays an important role in the central control of opioids with respect to the counter-regulatory response, although additional brain areas are likely involved in the other central effects that opioids can have on glucose metabolism.

Intra-NAC infusion of DAMGO activates a neural network that includes several brain areas associated with the control of glycaemia [24]. For example, intra-NAC infusion of DAMGO increases the activation marker cFos in the lateral hypothalamus [24], specifically in orexin expressing neurones [25]. Orexin neurones are implicated with the control of glycaemia because they are activated by hypoglycaemia [26] and activation of orexin neurones increases glucose production in the liver [27]. Another brain region that is activated upon intra-NAC infusion of DAMGO is the nucleus of the solitary tract (NTS) in the hindbrain [24]. This nucleus contains neurones

sensitive to changes in glycaemia and can modulate glucagon release [28]. In the present study, we observed both increased glycaemia and changes in plasma glucagon levels, although whether NTS neurones and lateral hypothalamic orexin neurones are involved remains to be determined in future experiments.

The brain can increase glycaemia via two main output mechanisms: either through activation of the hypothalamic-pituitary-adrenal axis, resulting in the release of corticosterone, or through an increase in sympathetic nervous system (SNS) activity [29]. In all but one of our experiments, intra-NAC DAMGO infusion increased corticosterone levels, although these increased levels did not consistently correlate with the effects on glycaemia. When DAMGO was infused under basal conditions, corticosterone increased similarly to that when DAMGO was injected right before an ITT or GTT, although a difference in glycaemia was evoked only in the latter conditions. Therefore, corticosterone does not appear to be the crucial mediator for the observed changes in glycaemia in the ITT or GTT. Accordingly, corticosterone is not known to affect short-term fluctuations in glycaemia: multiple reports indicate that infusion of cortisol (the human variant of corticosterone) does not change glycaemia [30,31] or only mildly increases glycaemia after 4 h [32]. Likewise, we have previously shown that deep brain stimulation of the nucleus accumbens at a low frequency significantly increased corticosterone but had no effect on glycaemia [17]. Overall, we conclude that the increased corticosterone after intra-NAC DAMGO infusion is a side effect of DAMGO infusion, and is not likely to mediate the changes in glycaemia. Future experiments will aim to further decipher the exact role of corticosterone in the effects on glycaemia seen upon NAC opioid stimulation.

Another possible mediator of the increased glycaemia seen after DAMGO infusion is the SNS. The SNS can influence glycaemia by altering pancreatic hormone release, glucose uptake in muscle and/or glucose production in the liver [33]. Indeed, the increased corticosterone release could also be mediated by the SNS because SNS activation enhances adrenal sensitivity to adrenocorticotrophic hormone, thereby increasing corticosterone release [34]. Previously, the effects of i.c.v. DAMGO infusion on glycaemia have been shown to be dependent on SNS activity [14] and we therefore hypothesise that the NAC-dependent effects of DAMGO on glycaemia could be mediated by the SNS, which will have to be tested in future experiments.

Interestingly, we only observed changes in glycaemia when DAMGO was administered during an ITT or GTT. We found that, in the absence of a glycaemic challenge, intra-NAC infusion of DAMGO has no effects on glycaemia. The possible DAMGO-induced activation of the SNS will likely cause an increase in serum epinephrine levels. Interestingly, the hyperglycaemic effects of epinephrine are found to be largely a result of the inhibitory effect of epinephrine on insulin action [35] and are therefore observed to be more pronounced during hyperinsulinemic conditions (such as during an ITT, or GTT) [35,36]. For example, i.v. doses of epinephrine that only cause a mild increase in basal glycaemia will induce a much greater increase in glycaemia when administered before a GTT [36]. The effects of these increased levels of epinephrine specifically affect the counter-regulatory phase, but not the initial change in glycaemia during the ITT or GTT. Potentially, the bolus of insulin or glucose has more pronounced effects on glycaemia than the subtle changes in SNS output as a result of intra-NAC DAMGO administration. Timing appears to be crucial because no significant effects were observed when DAMGO was administered 20 min prior to the ITT. These findings are consistent with the known short half-life (15 min) of DAMGO [37].

Previously, we have shown that serotonin and dopamine transmission can modulate the glucoregulatory function of the NAC. Specifically, increasing NAC serotonin levels also raised glycaemia. Although intra-NAC DAMGO infusion does not appear to increase serotonin release in the NAC [38], the insulin that is increased during both the ITT and GTT could potentially mediate changes in serotonin release. In vitro application of insulin on a NAC slice preparation showed no effects on serotonin release [39], although another study reported that, in vivo, an i.v. injection of insulin does increase serotonin release in a number of brain areas [40]. Unfortunately, that previous study did not investigate the NAC, and thus the in vivo effects of insulin on NAC serotonin release are yet to be determined. Because a strong connection has been found between central serotonin and the counter-regulatory response to hypoglycaemia [40,41], a possible role for serotonin in the effects seen upon DAMGO infusion appears likely, and will have to be investigated further.

By contrast to the effects of intra-NAC DAMGO infusion, activation of dopamine receptor-1 neurones in the NAC lowers glycaemia [16]. The effects of DAMGO on NAC dopaminergic signalling are two-fold. On the one hand, DAMGO infusion increases extracellular dopamine in the NAC [42] but, because μ-opioid receptors are present on the same NAC neurones that dopamine binds to, it also lowers the activity of these dopamine receptor-1 expressing neurones (which are typically activated by dopamine) [43]. Because we find opposing effects of intra-NAC DAMGO infusion compared to NAC dopamine-related effects on glycaemia, we hypothesise that the increase in dopamine release seen after DAMGO infusion is outweighed by direct inhibitory effects of DAMGO on dopamine-receptor neurones. In line with this hypothesis, when both DAMGO and a dopamine receptor-1 agonist were applied to slice preparations, DAMGO overruled the effects of dopamine on the intracellular pathway activated by the dopamine receptor-1, causing an overall inhibition of this pathway [44].

Because activation of NAC μ-opioid receptors enhances the response to hypoglycaemia, it would be an interesting therapeutic target to further investigate in the context of hypoglycaemia unawareness in insulin-dependent type 1 diabetes mellitus patients, a phenomenon where the body's ability to adequately respond to hypoglycaemia is impaired [45]. Interestingly, i.v. administration of a μ-opioid receptor antagonist, naloxone, is being investigated for its therapeutic abilities to enhance the counter-regulatory response to hypoglycaemia [46-48]. Although it may appear to be surprising that both μ-opioid receptor agonism and antagonism can beneficially affect the counter-regulatory response, opioids

are known to act differently centrally compared to peripherally. For example, i.v. infusion of naloxone increases glycaemia [6], whereas it lowers blood glucose levels when administered i.c.v. [49]. Likewise, central μ-opioid receptor activation decreases insulin sensitivity [14], whereas peripheral μ-opioid receptor activation can improve insulin sensitivity [50]. Deciphering these differential central and peripheral effects of opioid stimulation will be crucial for the use of opioid signalling as a therapeutic target for T1DM patients.

Overall, we found that activation of NAC μ-opioid receptors enhances the glycaemic response after an ITT or GTT, without affecting basal glycaemia. We show for the first time that the NAC is involved in the central effects of opioids on glucose metabolism. Because the effects of μ-opioid receptor activation in the NAC are specific to the response to a glycaemic challenge, this highlights the possibility to further investigate the involvement of NAC μ-opioid receptors in the defective counter-regulatory response during hypoglycaemia unawareness.

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## CONFLICT OF INTEREST

We have no conflicts of interest to disclose.

## AUTHOR CONTRIBUTIONS

**Laura Koekkoek:** Conceptualisation; Investigation; Writing – original draft. **Leslie Eggels:** Investigation; Methodology. **Tess Kool:** Investigation. **Luna van der Gun:** Investigation. **Khalid Lamuadni:** Investigation. **Margo Slomp:** Investigation. **Charlene Diepenbroek:** Investigation. **Mireille Serlie:** Writing – review & editing. **Andries Kalsbeek:** Writing – review & editing. **Susanne E. la Fleur:** Conceptualisation; Supervision; Writing – review & editing.

## PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/jne.13036.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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