Activation of central opioid receptors determines the timing of hypotension during acute hemorrhage-induced hypovolemia in conscious sheep

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Abstract

After an initial compensatory phase hemorrhage reduces blood pressure due to a widespread reduction of sympathetic nerve activity (decompensatory phase). Here we investigate the influence of intracerebroventricular (ICV) naloxone (opioid-receptor antagonist) and morphine (opioid-receptor agonist) on the two phases of hemorrhage, central and peripheral hemodynamics and release of vasopressin and renin in chronically instrumented conscious sheep. Adult ewes were bled (0.7 ml/kg/min) from a jugular vein until mean arterial blood pressure (MAP) reached 50 mmHg. Starting 30 min before and continuing until 60 min after hemorrhage either artificial cerebrospinal fluid (aCSF), naloxone or morphine was infused ICV. Naloxone (200 µg/min but not 20 or 2.0 µg/min) significantly increased the hemorrhage volume compared to aCSF (19.5±3.2 vs 13.9±1.1 ml/kg). Naloxone also increased heart rate and cardiac index. Morphine (2.0 µg/min) increased femoral blood flow and decreased hemorrhage volume needed to reduce MAP to 50 mmHg (8.9±1.5 vs 13.9±1.1 ml/kg). The effects of morphine were abolished by naloxone at 20 µg/min.

It is concluded that the commencement of the decompensatory phase of hemorrhage in conscious sheep involves endogenous activation of central opioid receptors. The effective dose of morphine most likely activated µ-opioid receptors but they appear not to have been responsible for initiating decompensation since a) naloxone only inhibited an endogenous mechanism at a dose much higher than the effective dose of morphine, b) the effects of morphine was blocked by a dose of naloxone which by itself did not delay the decompensatory phase.
Keywords

Naloxone, morphine, intracerebroventricular, vasopressin, renin
**Introduction**

In conscious mammals the hemodynamic response pattern to acute hemorrhage consists of, at least, two phases (40). Initially, the arterial blood pressure is well maintained due to an increased baroreceptor-mediated vasoconstrictor drive and tachycardia (compensatory phase). If hemorrhage continuous beyond a critical point a widespread decrease in vascular resistance in combination with a decrease in heart rate causes the arterial blood pressure to fall (decompensatory phase).

Since Faden & Holaday demonstrated that intravenous administration of the non-specific opioid receptor antagonist naloxone increases arterial blood pressure in hemorrhagic shock (16) a number of studies have been conducted using naloxone as a treatment in sepsis and hemorrhage c.f. (6). The usefulness of naloxone in the clinical setting is still under evaluation (3) but the studies have revealed important information regarding the physiology of hemorrhage. Among other things it was found that naloxone prevented the decompensatory phase in conscious rabbits (4; 26), and that the site of action is in the brain (47). This, together with the observation that similar doses of naloxone do not have a hypertensive effect in normovolemic animals (41), suggests a central opioid mechanism for the initiation of the decompensatory phase. However, contradictory results have been obtained depending on species, dose and anesthesia (40). One major problem is that general anesthesia distorts and sometimes abolishes the normal response pattern to hemorrhage (17; 28; 36). In addition, anesthesia also severely alters cardiovascular responses to exogenously administered opioids (38). This makes it preferable to study the central mechanisms of the decompensatory phase in unanesthetized animals. Studies on central opioidergic mechanisms in hemorrhage, using conscious animals, have been made in rats, rabbits and sheep. In hypovolemic
rabbits the increase in arterial blood pressure by central naloxone was found to be mediated by increased sympathoadrenal activity and peripheral vasoconstriction (45). Naloxone postponed the fall in arterial blood pressure, by an unknown mechanism, in conscious rats subjected to hemorrhage (31). In the only study in conscious sheep centrally administered naloxone did not affect the cardiovascular responses to hemorrhage (5), but the dose was lower than that used in other species. Paradoxically also morphine, primarily a µ-opioid receptor agonist, given intracerebroventricularly (ICV) has been shown to delay or abolish the decompensatory phase (34). This indirectly suggests that naloxone exerts its effect in preventing the decompensatory phase by acting on δ- or κ-opioid receptors. However, this study was done in anesthetized rats and the question remains whether centrally administered morphine affects the cardiovascular response to hemorrhage in conscious animals. Considering the conflicting data concerning the significance of central endogenous and effects of exogenous opioids in hemodynamic control during bleeding and the difficulties associated with studies of the CNS mechanisms responsible for hemorrhagic hypotension in anesthetized animals we decided to investigate effects of different doses of centrally administered naloxone and morphine on the decompensatory phase of hemorrhage in conscious, unrestrained sheep. We hypothesised that central opioidergic mechanisms are involved in causing the decompensatory phase of hemorrhage in conscious sheep.

**Methods**

The experimental protocol was approved in advance by the regional ethics committee in Stockholm and adheres to “European Convention for Protection of Vertebrate Animals
used for Experimental and other Scientific Purposes” (Council of Europe No 123, Strasbourg 1985) as well as the “APS guiding principles in the care and use of animals”.

**Animals and surgical preparation**

Nine Adult Texel cross bred ewes weighing 49 to 79 kg (mean 64 ± 10 kg) were used. The animals were housed in individual cages where they were fed twice a day and had free access to water and a salt block. Within a minimum of seven weeks before commencement of experimentation the sheep were anesthetized and their carotid arteries were exteriorised and placed in cervical skin loops bilaterally. Standard surgical anesthesia included premedication with acepromazin (0.3 mg/kg iv), anesthesia induction with sodium thiopental injection (10 mg/kg iv) followed by succinylcholine (1 mg/kg iv) and maintenance with isoflurane (2.1-2.3 % endtidal concentration) in an O₂/air mixture (40/60) via a respirator. After two weeks of recovery, the animals were anesthetized in the same manner and supplied with two permanent stainless-steel guide cannulae placed with their tip 2-3 mm above the lateral ventricles. In the final surgical preparation (after another two week recovery period) ultrasonic flow probes (Transonic System Inc, New York, USA) were placed unilaterally around the left renal and right femoral artery, respectively. Four animals were also, at the same time, equipped with a third flow probe around the superior mesenteric artery (cranial mesenteric artery in quadrupeds). Connecting tubes were tunneled subcutaneously and their connectors placed at a paralumbar position. Postoperative treatment for two days with buprenorphine (0.002 mg/kg im) and benzylpenicillin (20 000 IU/kg) / dihydrostreptomycin (0.0025 g /kg) were made routinely.
**Experimental preparation**

The experiments were performed with the animals standing in a cage in their habitual environment. Intravascular catheterisations were made at least 60 minutes before starting the experiments and under local anesthesia (lidocaine hydrochloride 5mg/ml). A cannulae (o.d. 1.0 mm) was inserted in one of the exteriorised carotid arteries for measurement of arterial blood pressure and blood sampling. A central venous catheter was introduced through the left jugular vein and was used for blood removal and retransfusion. Finally, a flow-directed thermodilution catheter (Swan-Ganz, Edward labs, Santa Ana, CA, USA) was fed into the pulmonary artery via an introducer in the right jugular vein and used for measuring mean pulmonary artery pressure (MPAP), central venous pressure (CVP), cardiac output (CO), and mixed venous oxygen saturation (SvO₂).

**Hemodynamic recordings**

The different blood pressures were continuously measured via pressure transducers (DPT-6003, PVB Medizin Technik, BMBH, Kirchseen, Germany). Signals from the thermodilution catheter were fed into a Vigilance® Edwards Critical Care Monitor (Baxter Healthcare Corporation, CA, USA) where the CO was calculated every 30 seconds and SvO₂ continuously. The flow probes were connected to two Transonic T208 two channel flow-meters and renal- (RBF), femoral- (FBF) and superior mesenteric blood flow (SMBF) were recorded continuously. Continuous on-line data acquisition was achieved by using the MP150/Acknowledge 3.7.2 system (BIOPAC Systems; Goleta, CA, USA) with a sampling rate of 100 Hz. Heart rate (HR) and mean arterial pressure (MAP) were computed from the arterial blood pressure signal and displayed on-line.
ICV-infusions

ICV-infusions were made by lowering a probe of suitable length into one of the permanently placed guide tubes. Free communication with the cerebrospinal fluid was assured by gentle siphoning of an aCSF-filled tube. For infusion another silicone tube connected the probe to a 5 ml syringe placed in an infusion pump (802 Syringe pump, Univentor, Zejtun, Malta). The ICV infusion rate (20 µl/min) corresponds to 15-25% of the total CSF production rate in sheep (37).

Morphine chloride (AstraZeneca, London, United Kingdom) or naloxone hydrochloride (Tocris, Bristol, United Kingdom) were dissolved and diluted to the required concentrations in sterile artificial cerebrospinal fluid (aCSF). The aCSF solution had the following composition (in mM): Na⁺ = 150; K⁺ = 2.9; Ca²⁺ = 1.1; Mg²⁺ = 0.9; Cl⁻ = 155; HCO₃⁻ = 24; HPO₄²⁻/H₂PO₄⁻ = 0.5. pH = 7.39.

Experimental protocol

The protocol for the experiments is illustrated in Fig 1.

Dose titration experiments

The first experiments were made to establish the doses to be used in later experiments. The aim was to determine the lowest ICV dose of morphine and naloxone able to affect the occurrence of the decompensatory phase during a venous hemorrhage. Two sheep were each subjected to four hypotensive hemorrhages concomitant with different ICV-infusions, performed at 7-day intervals. The procedure was similar to that used in the main protocol (see Fig 1 and below). The ICV-infusions (20 µl/min) were: aCSF (control) and morphine at doses of 0.2, 2.0 and 20 µg/min, respectively (Sheep 1).
aCSF (control) and naloxone at a dose of 2.0, 20 and 200 µg/min respectively (Sheep 2).

**Main experiments**

The animals were subjected to an ICV-infusion (20 µl/min) of either aCSF (control; n=6), morphine 2.0 µg/min (MOR; n=6), naloxone 2.0 µg/min (NLX 2; n=4) or naloxone 200 µg/min (NLX 200; n=6) before, during and after a hypotensive hemorrhage (Fig 1). The doses of morphine and naloxone were based on previous experiments in sheep (naloxone 2.0 µg/min) (5) and dose titration experiments (morphine 2.0 µg/min and naloxone 200 µg/min).

All ICV-infusions were preceded by a 10 min hemodynamic sampling period. Circulatory effects of the infusion *per se* were followed for 30 min before hemorrhage commenced. Thereafter, blood was aspirated from the left jugular cannulae at 0.7 ml/kg/min until the decompensatory phase occurred, defined as a sudden drop in MAP to 50 mmHg. Recovery was studied for 60 min followed by retransfusion. The ICV-infusions were stopped at the beginning of retransfusion (total infusion time: 90 min + hemorrhage time). Blood samples (17ml of venous blood and 2 ml of arterial blood) were drawn at five separate occasions: before the infusion started, at start of hemorrhage, at end of hemorrhage, 30 minutes after hemorrhage was stopped and finally 60 minutes after hemorrhage was stopped. The minimum interval between experiments was seven days.

**Additional experiments**

A separate experiment was performed in one sheep to 1) rule out any hypotensive effects of the current dose of morphine and 2) further confirm the hemodynamic effects.
of morphine per se. The animal was prepared in the same manner as in the main experiments and a 60 minute recovery period after connecting the catheters and probes was allowed. An ICV-infusion (20 µl/min) of morphine (2.0 µg/min) was started after 10 min of baseline registration and was discontinued after 150 min. Hemodynamic recordings were performed and the same parameters as in the main experiments were measured.

To ensure that the hemodynamic effects of morphine were elicited by activation of opioid receptors an additional experiment was performed. The protocol was equivalent to the main experiments apart from the type of ICV infusion. The animal was given an ICV infusion of naloxone only (1.0 mg/ml; 0.3 ml in 10 min) before commencement of an ICV infusion (20 µl/min) of morphine (0.1 mg/ml) and naloxone (1.0 mg/ml).

**Blood and plasma analyses**

The venous blood was portioned into pre-chilled tubes with heparin, respectively, EDTA as anticoagulants and centrifuged at + 4°C (3000 rpm). Plasma aliquots were stored at –20°C until assayed for vasopressin concentration and renin activity. Other portions were taken for determination of hematocrit, plasma osmolality (Auto & Stat Om 6010 osmometer; Kagaku Co, Japan) and protein concentration by refractometry (Atago Co, Japan). The carotid blood samples were used for immediate arterial blood gas analyses and determinations of sodium and potassium concentrations (ion optodes using ion-selective ionophores) performed on an Opti Critical Care analyser (AVL, Roswell, Georgia, USA).

Commercial radioimmunoassay kits were used for determinations of plasma vasopressin concentration (Euria-Vasopressin; Euro-Diagnostica, Malmo, Sweden) an plasma renin
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activity (Rianen Angiotensin I $^{[125I]}$ RIA kit, New England Nuclear, DuPont Scandinavia, Sweden).

**Calculations and statistical analysis**

Cardiovascular parameters were averaged off-line using a moving window of 2500 data points (25 seconds). Each value in the window was replaced with the mean value of the whole window. Renal-, femoral- and superior mesenteric arterial resistances (RVR, FVR, and SMVR) were calculated by dividing the difference between MAP and CVP by the renal-, femoral- and superior mesenteric blood flow, respectively. Cardiac output was divided by body weight to obtain cardiac index. Statistical analyzes were performed with Statistica 7.1 (Statsoft, Inc.). Data are expressed as means ± standard deviations (SD) or as confidence intervals (CI). Differences in hemorrhage volumes between groups were evaluated with one-way analysis of variance (ANOVA). Changes in parameters over time were analysed according to a two-way repeated measures ANOVA, the repeating variable being time. If the overall $F$-ratio was significant, Tukey’s post hoc test was used for comparisons of means. In case of significant interaction between time and ICV-infusion simple main effects were examined. As this procedure consists of multiple testing, the $p$-values were adjusted according to Bonferroni (1; 24). Differences were considered significant at $p \leq 0.05$. 
**Results**

The animals showed no signs of obvious discomfort during the experiments. When the decompensatory phase occurred the sheep usually calmly lied down. The respiratory rate was not monitored but it was noted to increase in association with the occurrence of hypotension.

The baseline plasma concentrations of $\text{Na}^+$, $\text{K}^+$, and protein as well as plasma osmolality and hematocrit were all within physiological limits in all groups. Blood gases, pH and base excess did not change significantly during the course of the experiment in any of the groups. However, there was tendency towards a respiratory alkalosis (increase in pH and decrease in pCO2).

**Dose titration experiments**

Typical MAP responses to the different doses of morphine and naloxone are illustrated in Fig 2. Morphine, at the rate of 2.0 $\mu$g/min and 20 $\mu$g/min, decreased the hemorrhage volumes needed to induce decompensation compared to control (Fig 2, panel A). Naloxone (200 $\mu$g/min) increased the hemorrhage volumes needed to induce decompensation compared to control (Fig 2, panel B).

**Effects of ICV naloxone and morphine on the appearance of decompensation**

Hemorrhage volumes needed to reduce MAP to 50 mmHg, i.e. induce decompensation are shown in Fig 3. Statistical evaluation revealed a highly significant main effect for ICV infusion treatment, $F(3,18) = 25.7$, $p<0.001$. Compared to control (aCSF ICV), 37 % less blood loss was required for decompensation to occur in sheep treated with
morphine ICV (2.0 μg/min) (8.9 ± 1.5 ml/kg, n= 6 vs 13.9 ± 1.1 ml/kg, n=6, p<0.001). This effect of morphine at 2.0 μg/min was abolished by concomitant infusion of naloxone at a dose of 20 μg/min. (Fig 2A).

Naloxone ICV had the opposite effect compared to that of morphine, but only in the animals treated with the highest dose (200 μg/min). With this dose of naloxone the hemorrhage volumes needed to induce hypotension was on average 40 % larger than in control animals (19.5 ± 3.1 ml/kg, n=6 vs 13.9 ± 1.1 ml/kg, n=6, p<0.001). The low dose of naloxone (2.0 μg/min) ICV did not affect the onset of decompensation in comparison to control animals (13.1 ± 2.0 ml/kg, n=4 vs 13.9 ± 1.1 ml/kg, n=6, p=0.96).

The average hemorrhage time for the different treatments was as follows: 13 min (MOR), 19 min (aCSF), 20 min (NLX 2.0) and 28 min (NLX 200).

**Cardiovascular effects of ICV naloxone and morphine per se**

At baseline there were no significant cardiovascular differences between groups. In control animals (aCSF ICV) and the NLX 2.0-group all cardiovascular parameters measured remained unchanged during the pre-hemorrhage infusion period. In contrast, the higher dose of naloxone (200 μg/min) significantly affected MAP, cardiac index and HR (Table 1 and Fig. 4). On average, MAP increased by 10 % (mean 9.1 mmHg, 95 % CI 3.9 to 14.3, n=6, p = 0.002), cardiac index by 16% (mean 14.1 ml/min/kg, 95 % CI 1.1 to 27.1, n=6 p = 0.03) and HR by 17% (mean 13 bpm, 95 % CI 8 to 18, n=6, p<0.001) in response to this dose of naloxone. Morphine ICV (2.0 μg/min) had no effect on blood pressures (MAP, MPAP, CVP), cardiac index, SVR, SvO₂, HR, RBF or SMBF. However, it caused a marked vasodilation of the femoral artery; FVR was reduced by 33 % (mean 0.35 mmHg/ml, 95 % CI 0.06 to 0.63, n=6, p = 0.02) (Fig 5),
increasing the blood flow from an average of $90 \pm 37$ ml/min to $145 \pm 49$ ml/min ($p = 0.02$). Naloxone at 20 $\mu$g/min abolished the effect of morphine on FVR ($n=1$). The decrease in FVR and increase in FBF was also evident in the single experiment where morphine 2.0 $\mu$g/min was infused ICV without concomitant hemorrhage. As illustrated in Fig 6, there was a progressive vasodilation during the first 50 min of infusion until the blood flow stabilized just below 200 ml/min. MAP remained at control levels during the entire infusion period.

**Cardiovascular responses to hemorrhage**

Representative, original tracings from one animal illustrating the MAP and HR responses to hemorrhage when subjected to the different ICV-infusions are shown in Fig 7. In all three experiments the two different phases of hemorrhage are easily detectable in both MAP and HR recordings, *i.e.* MAP was maintained together with an increase in HR until the decompensatory phase was elicited when both MAP and HR fell. Also, the effects of morphine (reduction) and naloxone (200 $\mu$g/min) (increase) on hemorrhage volume necessary to induce decompensation are evident.

In Figs 4 and 5 the cardiovascular responses to hemorrhage are not plotted on a time-scale. Since there is a significant individual variation in hemorrhage tolerance expressing the cardiovascular parameters as a function of time or volume would make the transition from the compensatory and decompensatory phase difficult to distinguish. Instead, the individual values of each animal at different stages of hemorrhage are grouped according to ICV-infusion and expressed as mean and 95% confidence intervals. The animals are, however, at largely different degrees of hypovolemia at corresponding stages of hemorrhage (compare Fig 7).
In the NLX 200-group MAP was elevated at the beginning of hemorrhage but during the decompensatory phase it fell to the same level as controls (Fig 4). The characteristic maintenance and subsequent conspicuous drop in arterial blood pressure were not changed by any of the treatments.

HR increased in parallel with the level of hypovolemia in all groups. Naloxone (200 μg/min) postponed the decompensatory phase and augmented the tachycardia seen in the compensatory phase (Table 1). In addition, naloxone (200 μg/min) attenuated, but did not prevent, the fall in HR typical for the decompensatory phase (Table 1). SVR did not increase during the compensatory phase and did not differ between control animals and any of the treatment groups. Irrespective of ICV-infusion SVR fell at time of decompensation (Table 1).

Cardiac index was largely unaffected during hemorrhage, although in the NLX 200-group a statistical significant reduction was obtained (mean 17.0 ml/min/kg between 20% and 100% of total hemorrhage, 95 % CI 5.0 to 28.9, n=6, p=0.008) (Fig 4). However, in the minutes following onset of hypotension the cardiac index dropped significantly in all groups and remained reduced. Considering the rather slow response time of the equipment used to measure cardiac output it is likely that the substantial reduction in cardiac index coincided with the decompensatory phase.

In Fig 5 the resistance in three different vascular beds is illustrated. RVR remained unchanged during the compensatory phase. In the NLX 200-group RVR rose significantly (p=0.006, n=6) during the decompensatory phase (80-100% of hemorrhage, Fig 5). The apparent increase in RVR during decompensation in the other groups was not statistically significant.
There was no significant change in FVR during the compensatory phase. However, decompensation was associated with a reduction in FVR \((p=0.04, n=22)\) that was independent of ICV-treatment. Hemorrhage caused SMVR to increase progressively \((p=0.02, n=13)\) (Fig 4) until decompensation. Like FVR, SMVR fell significantly between 80% and 100% of hemorrhage \((p=0.02, n=13)\). Apart from influencing the timing of the decompensatory phase none of the treatments had an effect on FVR or SMVR during hemorrhage.

Hemorrhage progressively reduced CVP in all groups (Fig 4). However, at a blood loss of 8.9 ml/kg (i.e. the volume necessary to cause decompensation in the MOR-group) the fall in CVP was more pronounced in sheep treated with morphine compared to the NLX 200-group (mean 2.8 mmHg, 95 % CI 0.6 to 5.1, \(n=6, p=0.02\)). This might reflect a tendency for a more pronounced central hypovolemia in the morphine treated animals. The \(SvO_2\) and MPAP showed no intergroup differences. During hemorrhage both variables were well maintained (data not shown) until the decompensatory phase occurred which caused a reduction from \(74.1 \pm 0.9\%\) to \(57.8 \pm 1.4\%\) \((p<0.001, n=22)\) and from \(15.3 \pm 0.7\) mmHg to \(11.9 \pm 0.8\) mmHg \((p=0.004, n=22)\), respectively.

**Post-hemorrhage recovery**

**Hemodynamics**

Despite a larger degree of hypovolemia, animals treated with naloxone (200 \(\mu\)g/min) recovered faster with respect to MAP and 30 min following the end of hemorrhage MAP had increased significantly more than in the control-group (mean increase 13.6 mmHg, 95 % CI 0.5 to 26.8 mmHg, \(p=0.04, n=6\)) (Fig 4).
Controls and animals treated with morphine or the lower dose of naloxone (2.0 μg/min) did not have tachycardia during the course of recovery (Table 1). In the NLX 200-group, however, heart rate remained elevated for the remainder of the post-hemorrhage recording period (Table 1). As noted above, cardiac index fell and remained below baseline values after end of hemorrhage. There was a slight recovery during the last 30 min of the post hemorrhage recovery period (Fig 4). These changes did not differ between groups.

The vascular resistance tended to increase during 30 min following hemorrhage, except in the renal artery in the NLX-200 group and the femoral artery in the morphine-treated animals (Fig 5 and Table 1). In the latter group (MOR) no change in FVR was seen during the post-hemorrhage recovery period (Fig 5). Apart from this exception all vascular resistances were at baseline levels in all groups at the end of the experiment. CVP increased gradually but only animals in the MOR-group reached pre-hemorrhage levels. The recovery of MPAP and SvO2 were faster and at 60 minutes all groups were at baseline levels.

**AVP and PRA**

None of the infusions caused changes in PRA or P-AVP levels *per se* (Fig. 8). The plasma AVP concentration increased in response to hypotensive hemorrhage in all groups (*p*<0.001, *n*= 6 in groups aCSF, NLX 200, MOR, *n*=4 in NLX 2.0, Fig 8). In animals treated with naloxone (200 μg/min) the plasma level of AVP was significantly higher than in controls (+645 pmol/l, 95 % CI 380 to 910, *p*=0.03, *n*=6) at end of hemorrhage (Fig 8). Changes in PRA followed the same pattern; it increased during hemorrhage (*p*<0.05, *n*=6 in all groups) (with the exception of NLX 2.0; *p*=0.06, *n*=4)
and the high dose of naloxone (200 µg/min) augmented the response (+1.8 ng ANG I/ml/h, 95 % CI 0.7 to 2.9, p=0.04, n=6) (Fig 8).

During the post-hemorrhage recovery period the AVP and PRA levels in the treatment groups did not significantly differ from controls (Fig 8).

**Discussion**

In this study we demonstrate that ICV naloxone at 200 µg/min, but not 0.2 or 2.0 µg/min, are able to postpone the onset of the decompensatory phase of a continuous hemorrhage in conscious sheep and thus prolong the maintenance of blood pressure and blood flow to vital organs. Furthermore, it significantly attenuates the associated bradycardia and facilitates a more rapid recovery of arterial blood pressure. ICV morphine, on the other hand, causes the decompensatory phase to occur at an earlier stage of blood loss. That central opioid receptor blockade with naloxone is able to postpone the decompensatory phase in rats and rabbits is well known (2; 14) but has previously not been reported in sheep. The opposite effect obtained here by ICV morphine is also a novel observation in sheep and is in contrast to that observed in other species.

Hypotension and bradycardia are reported to occur at a blood loss corresponding to 20-30 % of the estimated blood volume in all mammalian species studied (40). The present results show that the sheep is no exception in this regard. As a ruminant the estimated blood volume in sheep is somewhat lower in relation to body weight (60 ml/kg) than in other mammals (43).
Effects of ICV naloxone

There are three well established different opioid receptor types in the CNS; μ, κ and δ. They are all G-protein coupled and control cell activity through ion-gating and by regulating intracellular Ca\(^{2+}\)-levels, adenylyl cyclase and the mitogen-activated protein (MAP) kinase cascade (25). Naloxone and morphine both show a significant higher binding affinity for μ-receptors than for κ- (10- and 20-fold, respectively) and δ-receptors (20-fold) (18). Opioid receptors are widespread throughout the CNS (27) and endogenous activation occurs in many situations, including hemorrhagic shock (32). Since opioid signalling in the brainstem and the hypothalamus also have implications for the neural control of blood pressure (9; 19) an attractive possibility is that endogenous opioids acting in the CNS are somehow involved in inducing the decompensatory phase. This question has been addressed in a number of studies, primarily made in rats and rabbits (for review see 23; 40). The extensive work of Ludbrook et al on the subject has shown that the decompensatory phase can be prevented by naloxone in conscious rabbits undergoing a simulated hemorrhage (14) and possibly via sites of action in the brain stem (45). Further investigations using selective opioid-receptor antagonists has lead to the hypothesis that circulatory decompensation depends on stimulation of δ-receptors in the brainstem (15) or the spinal cord (2). The results in the present study also suggest a role for the central opioid system in the decompensatory phase of hemorrhage in conscious sheep, since naloxone delayed the onset of bradycardia and hypotension and morphine had the opposite effect. Our data could be interpreted as reflecting modulation of μ-receptor mediated mechanisms since the antagonist naloxone as well as the agonist morphine has the greatest affinity for this receptor. However, considering the binding affinity and
selectivity of the drugs in combination with the high dose of naloxone needed to postpone circulatory decompensation it appears less likely that naloxone exerted its effect by blocking the µ-receptor. Also, naloxone at a dose ineffective by itself blocked the premature hypotension caused by morphine which shows that morphine operated via opioid receptors that are not activated by endogenous ligands during hemorrhage. The fact that ICV naloxone and morphine, in these experiments, had disparate cardiovascular effects; naloxone changed heart rate and cardiac index and morphine affected vascular resistance, further supports the idea that they did not act via identical cerebral targets. Thus, in sheep naloxone appears to act on δ- or κ-opioid receptors to delay decompensation. This is in accordance with studies in other species where naloxones effects have been shown to be mediated via δ-opioid receptor antagonism (15).

The bradycardia and hypotension typical for the decompensatory phase are due to a withdrawal of sympathetic vasoconstrictor drive to many vascular beds as well as an increased vagal outflow to the heart (40). Hence, it seems likely that there is an inhibition of presympathetic neurons in the brainstem and/or more rostral sites as well as an activation of cell groups located in the brainstem controlling parasympathetic activity to the heart (i.e. n ambiguus and the dorsal motor nucleus of the vagus).

Naloxone has been shown to reverse the attenuation of renal sympathetic nerve activity during hemorrhage by a central mechanism (47) but there is no evidence that it actually interacts with the neuronal circuitries responsible for the decompensatory phase. It is possible that ICV naloxone gives rise to an increased activity in presympathetic neurones by pathways anatomically and functionally separate from those responsible for sympathoinhibition during hemorrhage. The results in this study partly support such a
mechanism since HR and cardiac index were significantly elevated by a high dose of naloxone *per se*, indicating an increased sympathetic nerve activity and/or decreased vagal activity to the heart before hemorrhage. However, given that there is no rise in vascular resistances (neither systemically (SVR) nor in any of the monitored vascular beds; RVR, FVR, SMVR) or plasma vasopressin levels before hemorrhage, the change in autonomic nerve activity by ICV naloxone in sheep appears to be limited to the heart. During hemorrhage ICV naloxone prolonged the compensatory phase. The augmented increase in heart rate probably reflects a more pronounced hypovolemia but also the direct effect of naloxone. Presumably, the tachycardia helped counteract the diminished stroke volume and prevent a substantial decrease in cardiac output. However, it appears unlikely that the shifting of the decompensatory phase was solely dependent on naloxone’s cardio-stimulatory effects and not its ability to inhibit cerebral mechanisms normally initiating hypotension. First, it has been shown in man that the fall in blood pressure appears even though the bradycardia is prevented with atropine (33) *i.e.* hypotension is dependent on a decrease in peripheral resistance. Second, in the present study the peripheral vasodilation appears synchronous with the hypotension. If the effect of naloxone was to postpone hypotension via increase in heart rate, the decrease in total peripheral resistance would have occurred at similar hemorrhage volumes as in control animals.

Thus, we conclude that naloxone in high concentrations antagonised endogenous opioid mechanisms which unopposed are involved in causing the peripheral vasodilation typical for the decompensatory phase. It is notable that the typical biphasic response pattern to hemorrhage remained, meaning that naloxone was only able to influence the timing, not the characteristics of the decompensatory phase.
Recent studies using microinjections, extracellular recordings and immediate early gene expression have identified sites of importance for controlling efferent preganglionic sympathetic nerve activity such as the paraventricular nuclei, the caudal midline medulla, ventrolateral periaqueductal grey, and the rostral ventrolateral medulla (8; 10; 11; 20; 42). All these sites are situated close to the CSF compartment.

The increases in P-AVP concentrations and PRA by hemorrhage were augmented by the high dose of naloxone. It has been shown that peripherally administered naloxone augments AVP responses to several different stimuli (39). The inhibitory influence of opioids on the hormone release has been shown to be mainly κ-receptor mediated (46).

The augmented AVP response to hypotensive hemorrhage observed here during the highest dose of ICV naloxone may reflect removal of opioidergic inhibition of AVP release via κ-receptor inhibition. However, the more pronounced hypovolemia in these experiments, due to the delayed appearance of the decompensatory phase, is an equally likely explanation for the reinforced AVP (and PRA) response.

Activation of peripheral V(1a) receptors by vasopressin improves cardiovascular recovery after hemorrhage (22) and this, in combination with a reinforced PRA response and an attenuated sympatho-inhibition, might be the reason for the augmented post-hemorrhage recovery in the NLX 200-group. There is no evidence in this study to suggest a central opioid mechanism inhibiting PRA or release of AVP in normotensive and normovolemic sheep.

**Effects of ICV morphine**

The premature development of the decompensatory phase caused by morphine ICV, observed here, is in contrast to what have been previously reported in rats and rabbits (13; 34; 35). In those species circulatory decompensation is prevented by central
stimulation of µ-opioid receptors. The disparity could be attributable to the major species differences in the distribution of opioid receptors in the brain (30) and also the relative proportion of receptor subtypes in various brain regions (29).

The nervous mechanisms that trigger the transition between compensated- and decompensated hemorrhage remains unclear (12) but it is well established that the appearance of hypotension is strongly related to central hypovolemia (40). The inability of 2.0 and 20 µg/min naloxone ICV to extend the compensatory phase of hemorrhage suggest that µ-opioid receptors are not part of the CNS-mechanism involved in initiating bradycardia and hypotension. Hence, the effect of morphine observed here is not likely due to a reinforcement of an endogenous opioid mechanism activated by hemorrhage. Instead, it is possible that the cardiovascular effects of morphine ICV changed the stimulus for the reflex by producing a peripheral vasodilation (Fig 5 & 6), thus reducing central blood volume. Assessed in terms of CVP the morphine group had a tendency towards a more rapid decrease in central blood volume during hemorrhage compared to the control group (significant compared to NLX 200-group). The decrease in peripheral vascular resistance by morphine is consistent with what has been reported in humans, where the effect is substantial (7). Furthermore, intravenous morphine has repeatedly been shown to inhibit the baroreceptor-heart rate reflex in man and animals (21) and although our results do not clearly indicate such a mechanism by ICV morphine in sheep, it can not be ruled out as a contributing mechanism to the fall in blood pressure.

**Cardiac index and blood flows**

In contrast to other hemorrhage studies in conscious rabbits, dogs and humans we found that the cardiac index was well maintained in sheep during the compensatory phase. Stroke volume decreased owing to diminished venous return but this was compensated
by increased heart rate. A fall in cardiac index was not evident until the appearance of the decompensatory phase. This appears not to be the result of an erroneous measurement due to a slow response time of the cardiac output monitor since there were no major reductions in peripheral flows and SvO₂ concomitant to hemorrhage, suggesting a more or less unaffected cardiac index. Furthermore, the gradual decrease in cardiac index, well correlated to the degree of hypovolemia, which is typically seen in response to hemorrhage in anesthetised sheep has been recorded using the same equipment (17). Therefore, the time resolution of the cardiac output computer to calculate the cardiac index during hemorrhage in these experiments seems sufficient. Besides a modest increase in superior mesenteric vascular resistance no vasoconstriction was seen during the compensatory phase. Consequently, the hypovolemic conscious sheep initially appears to defend the blood pressure more by preserving cardiac output and less via peripheral vasoconstriction than that seen in other species.

Vascular resistance in the femoral- and superior mesenteric artery dropped when decompensation started, coherent with the typical sympathoinhibition seen during hemorrhage. In the renal circulation the opposite event takes place, an increase in vascular resistance, most prominent in the NLX 200-group. This is most likely not a neural mechanism since renal sympathetic nerve activity is known to decrease during decompensation. The levels of PRA in this study, on the other hand, increased during hemorrhage in correlation to the degree of vasoconstriction in the renal artery and angiotensin II are known to increase vascular resistance in the kidney in conscious sheep (44). This indicates that a humoral factor counteracts the diminished sympathetic nerve activity to kidney vessels but not to the arteries in the gut or hindlimb.
Conclusion

Endogenous activation of central nervous system opioid receptors initiates hypotension and bradycardia during hemorrhage in conscious sheep. In accordance with previous studies in other species these opioid receptors appear to be of the κ- and/or δ-type. Opioid receptor blockade also results in increased cardiac performance. The results in this study suggest species differences in response to exogenous activation of central μ-opioid receptors during hemorrhage since results from studies in rats and rabbits show that this abolishes hemorrhage-induced hypotension and bradycardia. In sheep, these events are provoked by ICV morphine, possibly through peripheral vasodilation.

In addition it is noted that the compensatory phase of hemorrhage in conscious sheep rely mainly on mechanisms counteracting hypovolemia-induced reductions in cardiac output.

Acknowledgements

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Reference List


42. **Schadt JC, Shafford HL and McKown MD.** Neuronal activity within the ventrolateral periaqueductal gray during simulated hemorrhage in conscious rabbits. *Am J Physiol Regul Integr Comp Physiol* 00374, 2005.


Figure legends

Fig 1

Experimental protocol for the dose titration- and main experiments.
An intracerebroventricular (ICV) infusion of either artificial cerebrospinal fluid (aCSF), morphine or naloxone was started following baseline cardiovascular recordings. The doses of morphine and naloxone, and the number of experiments, are presented for the four different treatments in the main experiment. After 30 min of infusion a venous hemorrhage commenced and continued until the mean arterial blood pressure had dropped to 50 mmHg. Thereafter the animals were allowed to spontaneously recover for 60 min before the ICV-infusion was discontinued and the blood retransfused.

Fig 2

Blood pressure responses to hemorrhage in two sheep (panel A and B, respectively) subjected to ICV naloxone and/or morphine infusions.
The ICV-infusion (20 μl/min) commenced 30 min before a slow (0.7 ml/min/kg) venous hemorrhage was started. The hemorrhage was stopped when the blood pressure dropped to 50 mmHg.

Fig 3

Hemorrhage volumes needed to reduce mean arterial pressure to below 50 mmHg in response to different ICV-infusions: Artificial cerebrospinal fluid (aCSF; filled squares, n=6), morphine 2.0 μg/min (MOR; open circles, n=6), naloxone 2.0 μg/min (NLX 2.0;
filled triangles, n=4) and naloxone 200 µg/min (NLX 200; open triangles, n=6). Values are expressed both as individual hemorrhage volumes and as means together with 95 % confidence intervals. MOR and NLX 200 was statistically different from control (aCSF), p<0.001 for both groups.

**Fig 4**

Summary of the effects of intracerebroventricular-infusions on mean arterial blood pressure (MAP; upper panel), cardiac index (CI; middle panel) and central venous pressure (CVP; lower panel) before, during and after a hypotensive hemorrhage in conscious sheep. Infusion treatments, symbols and number of observations as in Fig 3. Naloxone 200 µg/min significantly increased MAP and cardiac index before hemorrhage commenced (ANOVA, p=0.002 and p=0.03, respectively) and also reinforced the MAP-recovery after hypotension (ANOVA, p=0.04). Note that the variables during hemorrhage are plotted against percentage of total blood loss, meaning that each individual are at different degrees of hypovolemia at all stages. Symbols show mean and 95 % confidence intervals.
**Fig 5**

Summary of the effects of different intracerebroventricular infusions on peripheral vascular resistances. The following infusions were made before, during and after a hypotensive hemorrhage: artificial cerebrospinal fluid (aCSF; filled squares), morphine 2.0 μg/min (MOR; open circles), naloxone 2.0 μg/min (NLX 2.0; filled triangles) and naloxone 200 μg/min (NLX 200; open triangles). Renal vascular resistance (RVR; upper panel) and femoral vascular resistance (FVR; middle panel): n= 6 for aCSF, MOR and NLX 200, n=4 for NLX 2.0. Superior mesenteric vascular resistance (SMVR; lower panel): n=4 for aCSF, n= 3 for MOR, NLX 2.0 and NLX 200. Note that the variables during hemorrhage are plotted against percentage of total blood loss, meaning that each individual are at different degrees of hypovolemia at all stages. Symbols show mean and 95 % confidence intervals. Statistical evaluation is presented in the results section.

**Fig 6**

Original recordings in one conscious sheep showing the effects of ICV morphine (2.0 μg/min) on mean arterial blood pressure (MAP), femoral vascular resistance (FVR) and femoral arterial blood flow (FBF) without concomitant hemorrhage. The dashed line represents the start of ICV-infusion (20 μl/min).

**Fig 7**

Blood pressure and heart rate responses to hemorrhage in one sheep subjected to different intracerebroventricular (ICV) infusions. 1) ICV morphine (2.0 μg/min), 2) ICV
artificial cerebrospinal fluid (aCSF) and 3) ICV naloxone (200 µg/min). The infusion was started 30 min prior to the hemorrhage.

**Fig 8**

Plasma renin activity (PRA) and vasopressin (AVP) in response to hemorrhage and ICV naloxone or morphine. Effects of intracerebroventricular-infusions (20 µl/min) of artificial cerebrospinal fluid (aCSF; filled squares; n=6), morphine 2.0 µg/min (MOR; open circles; n=6), naloxone 2.0 µg/min (NLX 2.0; filled triangles; n=4) and naloxone 200 µg/min (NLX 200; open triangles; n=6) on plasma renin activity (PRA) and plasma vasopressin levels before and after a hypotensive hemorrhage in conscious sheep. Blood samples were collected before the ICV-infusion started (baseline), after 30 min of infusion, directly after hemorrhage and 30 and 60 min post-hemorrhage. Symbols show mean and 95 % confidence intervals. * Indicates p<0.05 NLX 200 vs aCSF.
Table 1

Title: Heart rate and systemic vascular resistance in conscious sheep subjected to hemorrhage and an ICV infusion of artificial CSF, morphine or naloxone.

Legend: Effects of intracerebroventricular-infusions (20 µl/min) of artificial cerebrospinal fluid (aCSF), morphine 2.0 µg/min (MOR), naloxone 2.0 µg/min (NLX 2.0) and naloxone 200µg/min (NLX 200) on heart rate (HR) and systemic vascular resistance (SVR) before, during and after a slow (0.7 ml/min/kg) hypotensive hemorrhage in conscious sheep. HR and SVR during hemorrhage are expressed as the maximum value during the compensatory phase of hemorrhage and the minimum value during the decompensatory phase of hemorrhage. The infusion started 30 min before hemorrhage and lasted during the whole experiment. The hemorrhage was stopped when the mean arterial blood pressure was reduced to 50 mmHg. Values are mean ± SD. See methods for calculation of systemic vascular resistance (SVR). * p<0.05 for difference from baseline. † p<0.05 for difference from control (aCSF).
Central opioids and hemorrhage in conscious sheep

Fig 1

- aCSF, 20 μl/min ICV; n = 6
- Morphine 0.1 mg/ml, 20 μl/min ICV; n = 6
- Naloxone 0.1 mg/ml, 20 μl/min ICV; n = 4
- Naloxone 10 mg/ml, 20 μl/min ICV; n = 6

Fig 2

1A morphine 2.0 μg/min
2A morphine 20 μg/min
3A aCSF
4A morphine 0.2 μg/min
5A morphine 2.0 μg/min + naloxone 20 μg/min

1B aCSF
2B naloxone 2.0 μg/min
3B naloxone 20 μg/min
4B naloxone 200 μg/min
Fig 3
Fig 4

![Graph showing MAP, CI, and CVP over time during hemorrhage in conscious sheep.](image)
Fig 5
Fig 6
Central opioids and hemorrhage in conscious sheep

Fig 7

![Graph showing changes in MAP (mmHg) and HR (BPM) with hemorrhage (ml/kg).]

- MAP (mmHg) decreases with hemorrhage.
- HR (BPM) increases with hemorrhage.
Fig 8

- **PRA (ng ANG /ml/h):**
  - Baseline
  - Start hem
  - Stop hem
  - Stop hem +30
  - Stop hem +60

- **P-AVP (pmol/l):**
  - Baseline
  - Start hem
  - Stop hem
  - Stop hem +30
  - Stop hem +60

* denotes statistically significant difference.
### Table 1

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