Morphine withdrawal increases expression of GABA<sub>A</sub> receptor ε subunit mRNA in locus coeruleus neurons

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INTRODUCTION
The locus coeruleus (LC) is a bilateral nucleus in the brain stem consisting mostly of noradrenergic neurons. LC neurons send extensive projections throughout the brain, including the cerebral cortex, hippocampus, cerebellum and spinal cord. The activity of these neurons regulates attentional, physiological and behavioural processes and has a functional role in learning and memory [1]. Furthermore, the LC is a major component of the stress system [2]. Physical dependency on opiates is associated with withdrawal symptoms, which may be partly caused by a hyperactivity of the LC [3]. Increased activity of the LC neurons may be one of the major factors linked to opiate withdrawal [4]. After chronic morphine treatment, the medullo-coeruleal pathway undergoes transmitter-specific neuroadaptations [5], which may contribute to the hyperactivity of LC neurons during opiate withdrawal.

The noradrenergic neurons of the LC are under the inhibitory control of GABA [6], the major inhibitory transmitter in the mammalian CNS. Its fast inhibitory effects are mediated mainly through the ionotropic GABA<sub>A</sub> receptors [7]. The GABA<sub>A</sub> receptor possesses a heteropentameric structure comprised of any 16 different subunits (in mammals: α1–6, β1–3, γ1–3, δ, π, ε and θ, categorised according to sequence homology [8,9]) that are assembled in a poorly understood stoichiometry. Different subunit combinations (receptor subtypes) possess different pharmacological properties, and this seems to be the case also with the novel ε and θ subunit-containing receptors. α1β1γε GABA<sub>A</sub> receptor subtypes are modulated by pentobarbital and the neurosteroid 5α-pregn-3α-ol-20-one, but, unlike α1β1γ2S receptors, are insensitive to the benzodiazepine flunitrazepam [10]. In addition, ε-containing receptors can be directly activated by a number of general anaesthetic agents applied at high concentrations [11]. The αβεθ subunit combination displays up to a 4-fold decrease in affinity for GABA compared to the αβγθ subunit combination, and the binding of benzodiazepines to θ-containing receptors has not yet been confirmed [9].

Interestingly, the GABA<sub>A</sub> receptors located on LC neurons possess distinctive pharmacological characteristics, since they are poorly facilitated by benzodiazepines. The insensitivity of these receptors to diazepam and to the direct action of pentobarbital [12], combined with the reported lack of α1 and γ2 subunit gene expression in the LC, suggests the presence of a unique receptor subtype [13]. Recently, two novel subunits, ε and θ, have been shown to be highly enriched in the LC neurons [9,14]. These cells characteristically express tyrosine hydroxylase (TH) which is the rate-limiting enzyme in the biosynthesis of catecholamines [15].

Opiate withdrawal is associated with increased LC neuronal activity. The LC neurons are normally under GABAergic inhibition, most likely via selective GABA<sub>A</sub> receptor subtypes. To understand how the GABA<sub>A</sub> receptor ε and θ subunits, enriched in the LC, might be regulated by the increased neuronal activity, we investigated, using in situ hybridisation, the mRNA expression of these subunits in response to naloxone-precipitated morphine withdrawal.

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MATERIALS AND METHODS

Animals: All animal experiment protocols were approved by the Western Finland provincial government (LSLH-1999-5429/Ym-23). Adult male Sprague–Dawley rats (weight 320–400 g) were habituated to the animal facility and housed 3/cage in Macrolon-3 (22.5 × 39 × 18 cm) cages with aspen chip bedding. The rats were randomly assigned to three treatment groups (n = 10): morphine withdrawal, chronic morphine treatment and saline controls. In addition, four home cage control animals were studied in parallel. In the morphine withdrawal group, opiate dependence was induced by i.p. injections of escalating doses of morphine hydrochloride (RBI, Natick, MA, USA) three times a day for 5 days. The morphine treatment regime was day 1: 3 × 10 mg/kg; day 2: 2 × 10 mg/kg and 1 × 20 mg/kg; day 3: 2 × 20 mg/kg and 1 × 40 mg/kg; day 4: 3 × 40 mg/kg; day 5: 2 × 80 mg/kg and 1 × 100 mg/kg. Doses of 10–40 mg/kg were injected in a volume of 1 ml/kg, and those of 80 and 100 mg/kg in 2 ml/kg. The first injection was given between 09:00 and 10:00 h, the second between 14:00 and 15:00 h and the third between 20:00 and 21:00 h. This treatment protocol has been shown to produce significant opioid dependence [16]. On the 6th day (i.e. 24 h after the last morphine injection) opioid withdrawal was precipitated by an i.p. injection of naloxone hydrochloride (5 mg/kg, RBI). Morphine and naloxone were diluted in saline. The chronic morphine treatment group received morphine injections according to a treatment regime identical to that of the withdrawal group, but received no naloxone injection. The saline control group received saline injections three days a day for 5 days and on the 6th day they were treated with naloxone.

Behaviour of the animals was recorded for 15 min with a video recorder after the last injection in individual cages to observe the withdrawal symptoms. The behavioural parameters recorded were the presence or absence of screaming, shaking, nose bleeding, defecation, urination and redness of the eyes. Thereafter, the rats were returned to their home cages.

In situ hybridisation: The animals were decapitated 2 h after they had received the last injection (morphine or naloxone) in the morphine withdrawal experiment. The experiment was carried out in two sets. The cage control animals were decapitated immediately after being transferred to the experimental room from their home cages in conjunction with the decapitation of the first set of experimental animals. Brains were removed and frozen immediately on dry ice and stored at −70°C until use. Brain coronal sections (14 μm) containing the LC were cut on a cryostat (Microm HM 500 OM; Microm Laborgeräte GmbH, Walldorf, Germany), mounted onto poly-l-lysine-coated glass slides and dried at room temperature for 2 h. Sections were then fixed in 4% paraformaldehyde, washed in phosphate-buffered saline for 5 min, dehydrated in 70% ethanol and placed in 95% ethanol for storage at 4°C until use. In situ hybridisation with 33P-labeled oligonucleotide probes was performed as described in detail by Sinkkonen et al. [14]. The oligonucleotide probes complementary to rat cDNA sequences were as follows [14]: TH probe was complementary to nucleotides 867–911 of rat cDNA (GenBank accession number M10244), GABA A receptor β subunit probe (nucleotides 2240–2275, AF189262), α subunit probe (1240–1284, L08490) and γ2 subunit probe (1170–1214, L08497). Specificity of the probes was confirmed with 100-fold excess of unlabeled probes (Fig. 1). After hybridisation at 42°C overnight, the sections were washed in 1× SSC at room temperature for 10 min and then in 1× SSC at 55°C for 30 min followed by alcohol dehydration by 3 min washing steps (1× SSC, 0.1× SSC, 70%, 95% EtOH). Sections were allowed to air-dry before exposure to Biomax MR film (Eastman, Kodak, Rochester, NY, USA) with 4C standards (Amersham, Buckinghamshire, UK) at 4°C. Exposure time was 2–4 weeks. Quantification of the in situ hybridisation signals in the LC and adjacent cerebellar granule cell layer was performed by computerised image analysis using an A1S image analysis device and software (Imaging Research, St. Catherines, Ontario, Canada). Brain regions were identified using an atlas of the rat brain [17]. For each animal and brain region of interest, at least two bilateral measurements were made on every brain section. Their average represented the probe density value for that specific brain region for that animal. Images from representative films were produced by scanning the films using an HP ScanJet 4c/T scanner and HP DeskScan II program (Hewlett Packard, Palo Alto, CA, USA) and Adobe Photoshop (version 3.0; Adobe Systems, Mountain View, CA, USA).

To verify the cellular location of mRNA expression in the LC region, we also dipped some parallel sections into emulsion (NTB-2 photographic emulsion, Kodak, Rochester, NY, USA), and exposed them in light-tight boxes at 4°C for 1–4 weeks. Slides were developed for 2 min in D-19 developer (Kodak) diluted 1:1 with water, rinsed in deionized water and fixed in Kodak fixer. After fixing, the slides were dried, lightly stained in 0.1% thionin (Sigma, St. Louis, MO, USA) solution for anatomical localisation, and mounted with Permount (Fisher, Pittsburgh, PA, USA) and glass coverslips. Silver grains were detected with a light microscope (Leica DMR, Leica Microsystems Wetzlar GmbH, Germany).

Statistics: Results were tested for statistical significance of the differences between groups by one-way ANOVA, followed by Tukey’s multiple comparison test as a post hoc test.

RESULTS

All animals of the naloxone-treated morphine withdrawal group presented 3–5 of the five signs of withdrawal syndrome (Table 1). The animals of the chronic morphine treatment group not treated with naloxone did not show any withdrawal signs and were actually identical in this respect to the animals of the saline control group that was also given naloxone. These results indicate that a strong opioid withdrawal state was achieved with the present protocol.

Strong and specific labeling of the LC neurons of the naloxone-treated morphine withdrawal group presented 3–5 of the five signs of withdrawal syndrome (Table 1). The animals of the chronic morphine treatment group not treated with naloxone did not show any withdrawal signs and were actually identical in this respect to the animals of the saline control group that was also given naloxone. These results indicate that a strong opioid withdrawal state was achieved with the present protocol.
below). The in situ hybridisation signal specific for TH mRNA was significantly different between the four experimental groups (F(3,30) = 4.24, p < 0.05), being elevated in the morphine withdrawal group (p < 0.05) in comparison with the other groups (Fig. 2), consistent with increased activity of LC noradrenergic neurons. Chronic morphine treatment did not cause any significant alterations in the TH mRNA signal. The GABA A receptor γ subunit mRNA signal also differed between the groups (F(3,30) = 16.78, p < 0.0001), being increased (p < 0.05) in the morphine withdrawal group compared with the other treatment groups (Fig. 2b), but the δ subunit mRNA did not show any significant differences between the groups (F(3,30) = 0.82, p > 0.4; Fig. 2b). We detected no difference in the expression levels of these subunits between the non-treated cage control, chronic morphine and saline/naloxone treatment groups.

The expression of γ2 subunit mRNA in the LC determined from films was extremely low, and there were no differences in expression levels seen in the morphine/naloxone group (10.2 ± 1.3 nCi/g, mean ± s.e., n = 10) when compared to chronic morphine (11.1 ± 0.4 nCi/g) and saline/naloxone groups (10.6 ± 0.2 nCi/g). The signal intensity of α1 subunit mRNA in the LC was not above the film background. There were no differences between the treatment groups in α1 and γ2 in subunit mRNA expression levels determined in the granule cell layer of the cerebellum (Fig. 3), indicating that opioid withdrawal does not produce global alterations in GABA A receptor subunit expression.

**DISCUSSION**

We describe here that opioid withdrawal increases the expression of TH and GABA A receptor ε subunit mRNAs in the LC shortly after induction of withdrawal by naloxone. No clear effect was found in the expression of the δ subunit, another GABA A receptor subunit for which the gene was recently found. No increases in the cerebellar

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**Table 1.** Naloxone-precipitated morphine withdrawal symptoms in rats treated chronically with escalating doses of morphine.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Screaming a</th>
<th>Shaking</th>
<th>Defecation b</th>
<th>Urination</th>
<th>Nose bleeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline/naloxone</td>
<td>0</td>
<td>0</td>
<td>1 (+)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Chronic morphine</td>
<td>0</td>
<td>0</td>
<td>1 (+)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Morphine/naloxone</td>
<td>10</td>
<td>10</td>
<td>10 (+++)</td>
<td>8</td>
<td>4</td>
</tr>
</tbody>
</table>

aThe number of animals (n = 10/group) showing each withdrawal sign during a 15 min period immediately after naloxone administration.
bThe level of defecation expressed as (+) (normal) to +++ (3-fold increase).
genes might be fairly similar in some cases, e.g. for α1, β2 and γ2 subunits [20,21], in most clusters the genes are regulated individually. Our data suggest that the promoter of the ε subunit gene might have regulatory elements that are sensitive to neuronal activation, e.g. via the action of immediate early genes, known to be activated in the LC neurons during opioid withdrawal [22]. The present data and those of Moragues et al. [23] do not provide evidence for coordinated expression of the genes clustered in the chromosome Xq28 [9] together with the ε subunit, i.e. α3 and θ subunit genes. However, as all these genes are expressed in the LC neurons, the receptor subunits they encode may form the predominant GABA_A receptor subtype responsible for GABAergic inhibition of these neurons.

Opioid withdrawal reactions are not usually life-threatening, but harmfully complicate any attempt to curtail the usage of opioid agonists [22]. Presently, withdrawal reactions are being treated with sedative α2 adrenoceptor agonists, e.g. lofexidine and clonidine, which unfortunately cause in many patients strong hypotension that limits the usefulness of these drugs [24]. Therefore, our data suggesting the presence of novel inhibitory GABA_A receptor subtypes in the LC neurons can be a start for developing more selective and tolerable drugs for treating overactive LC neurons.

**CONCLUSION**

Several studies have implicated increased activity of LC neurons in opiate withdrawal. Our data suggest that the GABAergic system adapts to changes in neuronal activity during naloxone-precipitated opioid withdrawal. More interestingly, our data point to an involvement for at least one (ε) of the two novel GABA_A receptor subunits, ε and θ, in functional modulation of the LC. This may be a starting point for the development of novel GABA_A receptor non-benzodiazepine site agonists, acting selectively on discrete

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**Fig. 2.** (a) In situ hybridisation signals for the expression of tyrosine hydroxylase (TH) mRNA and GABA_A receptor ε and θ subunit mRNAs in the LC nuclei in morphine-treated rats with and without naloxone-precipitated withdrawal reactions. (b) Optical density values of quantified in situ hybridisation signals are presented as nCi/g after calibration with radioactivity standards exposed on the same films. Results are given as means ± s.e. (n = 10). Statistical significance of the differences between the treatment groups was assessed by one-way ANOVA followed by Tukey’s test: *p < 0.05.
brain areas of major functional significance for normal and pathological activity.

REFERENCES

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