

Morphine withdrawal increases expression of GABA_A receptor ϵ subunit mRNA in locus coeruleus neurons

Anu T. Heikkilä, Oxana Echenko, Mikko Uusi-Oukari, Saku T. Sinkkonen and Esa R. Korpi^{CA}

Department of Pharmacology and Clinical Pharmacology, University of Turku, FIN-20520 Turku, Finland

^{CA}Corresponding Author

Received 26 June 2001; accepted 17 July 2001

An increase in the activity of brain stem locus coeruleus noradrenergic neurons has been hypothesised to be a major factor accounting for opiate withdrawal symptoms. These neurons are under GABAergic inhibition. Their GABA_A receptors have unique pharmacological properties, most likely due to the enriched expression of GABA_A receptor subtypes containing novel ϵ and θ subunits. Using *in situ* hybridisation of

cryostat sections, we now report a significant increase in the ϵ subunit mRNA expression after precipitation of opioid withdrawal by naloxone. Similar changes were detected in tyrosine hydroxylase mRNA expression. The results suggest increased formation of unique GABA_A receptor subtype(s) in the locus coeruleus neurons during increased neuronal activity. *Neuro-Report* 12:2981–2985 © 2001 Lippincott Williams & Wilkins.

Key words: GABA_A receptor subunits; *In situ* hybridisation; Morphine; Opiate withdrawal

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_A receptors [7]. The GABA_A receptor possesses a heteropentameric structure comprised of any 16 different subunits (in mammals: α 1–6, β 1–3, γ 1–3, 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. α 1 β 1 ϵ GABA_A receptor subtypes are modulated by pentobarbital

and the neurosteroid 5 α -pregnan-3 α -ol-20-one, but, unlike α 1 β 1 γ 2S 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 α β γ θ subunit combination displays up to a 4-fold decrease in affinity for GABA compared to the α β γ subunit combination, and the binding of benzodiazepines to θ -containing receptors has not yet been confirmed [9].

Interestingly, the GABA_A 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 α 1 and γ 2 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_A receptor subtypes. To understand how the GABA_A 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.

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.

Morphine withdrawal experiment: 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 times 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 ^{33}P -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 ϵ subunit probe (nucleotides 2240–2275, AF189262), θ subunit probe (86–130, AF189261), $\alpha 1$ subunit probe (1240–1284, L08490) and $\gamma 2$ 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 $1\times$ SSC at room temperature for 10 min and then in $1\times$ SSC at 55°C for 30 min followed by alcohol dehydration by 3 min washing steps ($1\times$ SSC, $0.1\times$ SSC, 70% EtOH, 95% EtOH). Sections were allowed to air-dry before exposure to Biomax MR film (Eastman, Kodak, Rochester, NY, USA) with ^{14}C 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 AIS image analysis device and software (Imaging Research, St. Catharines, 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 was observed by the probes for TH and ϵ and θ subunit mRNAs (Fig. 1). No labeling was observed for the $\alpha 1$ and $\gamma 2$ subunits in the LC neurons (data not shown), although these were expressed in the cerebellar granule cells (see

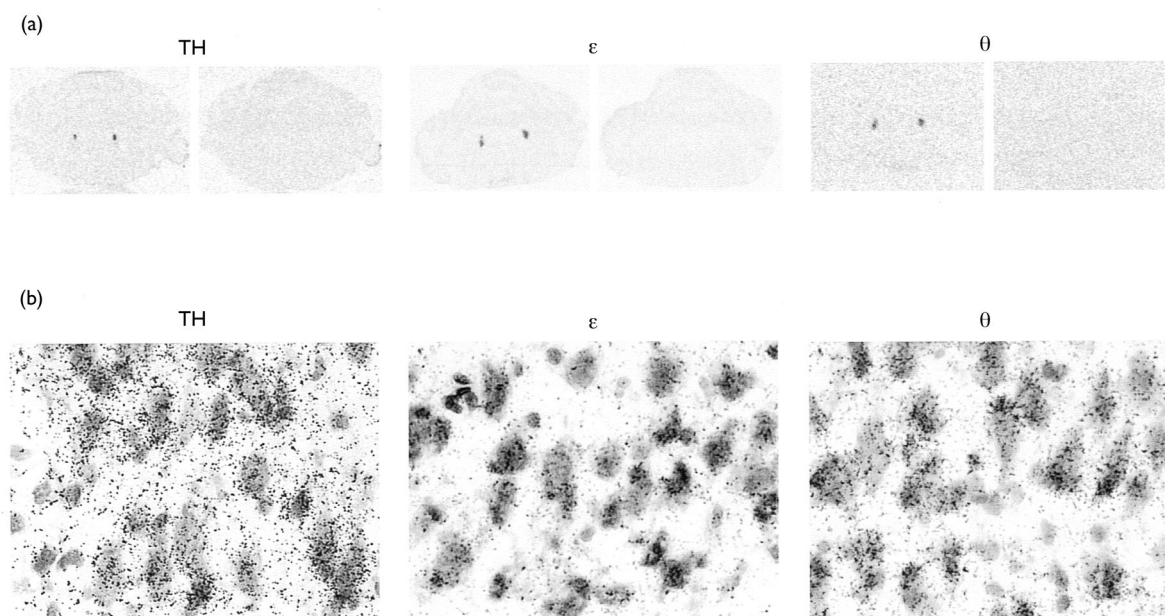


Fig. 1. Representative images of expression of tyrosine hydroxylase (TH) and GABA_A receptor ϵ and θ subunit mRNAs in LC neurons of control rats. (a) Specificity of the hybridisation signals of the probes with (left) and without (right) 100-fold excess of the corresponding unlabelled oligonucleotide probe. (b) Localisation of hybridisation at the cellular level. After hybridisation with specific oligonucleotide probes, the sections were dipped in photographic emulsion, exposed and developed. The images for all probes show enrichment of silver grains over thionin-stained cells, with little signal in the neuropil, indicating that the LC neurons express these mRNAs.

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

Treatment	Screaming ^a	Shaking	Defecation ^b	Urination	Nose bleeding
Saline/naloxone	0	0	1 (+)	0	0
Chronic morphine	0	0	1 (+)	0	0
Morphine/naloxone	10	10	10 (+++)	8	4

^aThe number of animals ($n = 10/\text{group}$) showing each withdrawal sign during a 15 min period immediately after naloxone administration.

^bThe level of defecation expressed as + (normal) to +++ (3-fold increase).

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 ϵ 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 θ 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 $\gamma 2$ 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 ($10.2 \pm 1.3 \text{ nCi/g}$, mean \pm s.e., $n = 10$) when compared to chronic morphine ($11.1 \pm 0.4 \text{ nCi/g}$) and saline/naloxone groups ($10.6 \pm 0.2 \text{ nCi/g}$). The signal intensity of $\alpha 1$ subunit mRNA in the LC was not above the film background. There were no differences between the treatment groups in $\alpha 1$ and $\gamma 2$ 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 θ subunit, another GABA_A receptor subunit for which the gene was recently found. No increases in the cerebellar

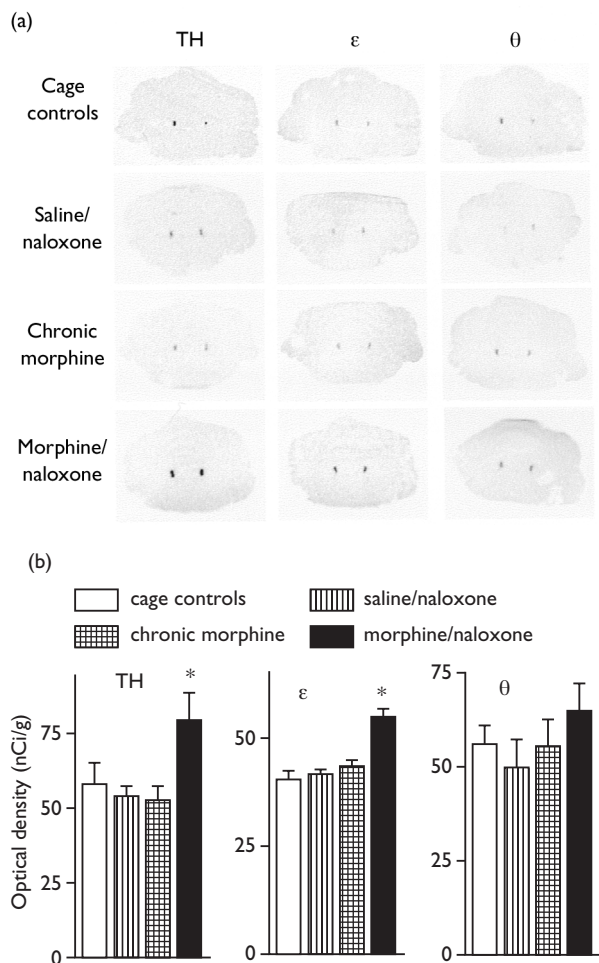


Fig. 2. (a) *In situ* hybridisation signals for the expression of tyrosine hydroxylase (TH) mRNA and GABA_A receptor ϵ and θ subunit mRNA in the LC nuclei in morphine-treated rats with and without naloxone-precipitated withdrawal reactions. (b) Optical density values of quantified *in situ* hybridisation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

granule cell layer (or no expression at all in the LC) were detected in the signals for the $\alpha 1$ and $\gamma 2$ subunits by opioid withdrawal, indicating the specificity of the observed alterations for TH and the ϵ subunit. The increased TH and ϵ subunit expressions were selective for the withdrawal reaction, since their levels were not altered by chronic morphine treatment or by naloxone injection in saline-treated animals. Therefore, opioid withdrawal is accompanied by specific alterations in GABA_A receptor subunit gene expression in the LC neurons.

It is known that the expression of TH is activity dependent and, therefore, its increased expression during opioid withdrawal is in agreement with strong activation of LC neurons [18]. The regulation of GABA_A receptor subunit genes is poorly understood. GABA_A receptor subunit genes appear in the genome as gene clusters [19], and although the regulation of the expression of clustered

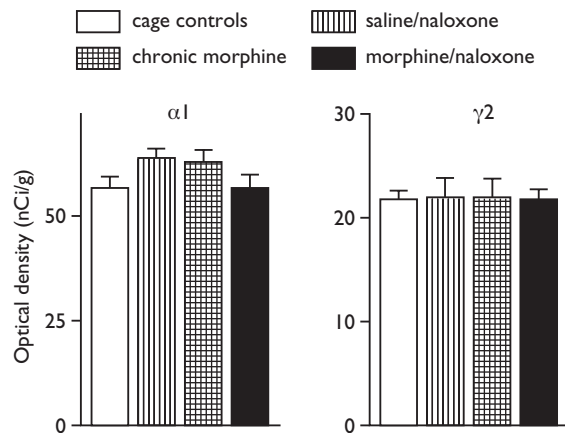


Fig. 3. Optical density values of quantified *in situ* hybridisation signals of GABA_A receptor $\alpha 1$ and $\gamma 2$ subunit mRNAs in the cerebellar granule cell layer of the serial sections of rats used for Fig. 2. The data are presented as nCi/g after calibration with radioactivity standards exposed on the same films. Results are given as means \pm s.e. (cage controls $n = 4$, other groups $n = 10$). There were no statistically significant differences between the groups (ANOVA for the $\alpha 1$ and $\gamma 2$ signals $F(3,30) = 2.05$, $p > 0.16$, and $F(3,30) = 0.008$, $p > 0.99$, respectively).

genes might be fairly similar in some cases, e.g. for $\alpha 1$, $\beta 2$ and $\gamma 2$ 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. $\alpha 3$ 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 $\alpha 2$ 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

brain areas of major functional significance for normal and pathological activity.

REFERENCES

1. Coull JT, Büchel C, Friston KJ *et al.* *NeuroImage* **10**, 705–715 (1999).
2. Chrousos GP and Gold PW. *JAMA* **267**, 1244–1252 (1992).
3. Rasmussen K, Beitner-Johnson DB, Krystal JH *et al.* *J Neurosci* **10**, 2308–2317 (1990).
4. Harris GC and Williams JT. *J Pharmacol Exp Ther* **261**, 476–483 (1992).
5. Van Bockstaele EJ, Peoples J, Menko AS *et al.* *J Neurosci* **20**, 8659–8666 (2000).
6. Ijima K and Ohtmo K. *Am J Anat* **181**, 43–52 (1988).
7. Hevers W and Lüddens H. *Mol Neurobiol* **18**, 35–86 (1998).
8. Barnard EA, Skolnick P, Olsen RW *et al.* *Pharmacol Rev* **50**, 291–313 (1998).
9. Bonnert TP, McKernan RM, Farrar S *et al.* *Proc Natl Acad Sci USA* **96**, 9891–9896 (1999).
10. Whiting PJ, McAllister G, Vasilatis D *et al.* *J Neurosci* **17**, 5027–5037 (1997).
11. Thompson SA, Bonnert TP, Whiting PJ *et al.* *Toxicol Lett* **100-101**, 233–238 (1998).
12. Chen CL, Yang YR and Chiu TH. *Eur J Pharmacol* **386**, 201–210 (1999).
13. Luque JM, Malherbe P and Richards J. *Mol Brain Res* **24**, 219–226 (1994).
14. Sinkkonen ST, Hanna MC, Kirkness EF *et al.* *J Neurosci* **20**, 3588–3595 (2000).
15. Ijima K, Mitsuru S, Naosuke K *et al.* *Anat Rec* **234**, 593–604 (1992).
16. Pineda J, Torrecilla M, Martin-Ruiz R *et al.* *Neuropharmacology* **37**, 759–767 (1998).
17. Paxinos G and Watson C. *The Rat Brain in Stereotaxic Coordinates*. 2nd Edition. New York: Academic Press; 1986.
18. Aston-Jones G, Shipley MT, Chouvet G *et al.* *Prog Brain Res* **88**, 47–75 (1991).
19. Russek SJ. *Gene* **227**, 213–222 (1999).
20. Wisden W, Laurie DJ, Monyer H *et al.* *J Neurosci* **12**, 1040–1062 (1992).
21. Uusi-Oukari M, Heikkilä J, Sinkkonen ST *et al.* *Mol Cell Neurosci* **16**, 34–41 (2000).
22. Nestler EJ. *Semin Neurosci* **9**, 84–93 (1997).
23. Moragues N, Ciofi P, Lafon P *et al.* *Eur J Neurosci* **12**, 4318–4330 (2000).
24. Cox S and Alcorn R. *Lancet* **8962**, 1385–1386 (1995).

Acknowledgements: The study was supported by the Academy of Finland (E.R.K.), CIMO (O.E., E.R.K.), Sigrid Juselius Foundation (E.R.K.) and the Research and Science Foundation of Farnos (A.T.H.).