Opiates increase the number of hypocretin-producing cells in human and mouse brain and reverse cataplexy in a mouse model of narcolepsy

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Greatly increased number of detected hypocretin cells in human heroin addicts

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One Sentence Summary: We find that human heroin addicts have 54% more hypocretin neurons than controls, that morphine can induce similar changes in mice and that morphine can reverse symptoms in narcolepsy, a disorder caused by hypocretin cell loss.

Abstract

The changes in brain function that perpetuate opiate addiction are unclear. In our studies of human narcolepsy, a disease caused by the loss of hypocretin (orexin) cells, we encountered a "control" brain with 50% more hypocretin cells than any prior control brain. We discovered that this individual was a heroin addict. We now report that human heroin addicts have, on average, 54% more immunohistochemically detected hypocretin producing cells than controls. Similar increases can be induced in wild type mice by longterm administration of morphine. The increased number of detected hypocretin neurons is not due to neurogenesis and outlasts administration of morphine by several weeks. The number of melanin concentrating hormone cells, which are in the same hypothalamic region as hypocretin cells, was not changed by morphine administration. Morphine administration restored the population of detected hypocretin cells to the normal level in hypocretin depleted mice, and eliminated or greatly decreased cataplexy in narcoleptic mice, suggesting that opiate agonists may have a role in the treatment of narcolepsy. Induction of specific longterm changes in peptide production, far outlasting the half-life of the administered drugs, may be useful in treating diseases characterized by neuronal loss. Our findings also indicate that some portion of the loss of specific cell types that have been reported in neurological diseases may be due to reduced production of the identifying label used for counting the neurons, rather than to neuronal death.

INTRODUCTION

Previously, we reported that human narcolepsy was linked to a loss of hypocretin cells, with an average 90% loss of immunohistochemically identified cells[1]. More recently, we reported a decreased number of immunohistochemically identified hypocretin neurons in Parkinson's disease[2, 3]. In analyzing control human brains for further studies of cell loss, we were surprised to encounter a brain with 50% more hypocretin neurons than we had found in any prior human brain. Further investigation revealed that this individual was a former heroin addict. We therefore acquired the brains of additional human heroin addicts to see if this was a consistent correlate of heroin addiction. .

We then examined the effect of morphine administration to determine if it produced a similar elevation in the number of hypocretin cells. Further, because narcolepsy is caused by the loss of hypocretin cells, we tested the effect of morphine administration on cataplexy, the unique symptom of narcolepsy.

The annual rate of opiate overdose deaths in the United States has increased fourfold in the last 17 years, with more than 300,000 deaths since 2000. Addicts are unable to overcome opiate addiction, even as they realize the destructive effect it is having on them and their families[4]. We discuss the present work in the context of studies suggesting that hypocretin cell activity is linked to pleasure-mediated arousal and hence may play a role in perpetuating addiction [5-7].

RESULTS

Increased number of immunohistochemically positive hypocretin (orexin) neurons in human heroin addicts

The figure legends contain statistical details. The numbers of subjects in each human and animal study are indicated in Tables 1 and 2.

There was an average 54% increase in the number of detectable hypocretin neurons in human heroin addicts relative to matched human controls (Figs 1a & c). Hypocretin cells were 22% smaller in the addicts (Fig 1b, 1d). Approximately 90% of hypocretin neurons in human control subjects also contain neuronal activity regulated pentraxin (Narp), a secreted neuronal pentraxin co-localized with hypocretin in hypothalamic neurons[8]. This percentage was similar in the larger number of hypocretin neurons observed in human heroin addicts (Fig 1e), suggesting that the transmitter contents of the cells in the addicts were similar in this respect to the cells in controls.

Dose dependent effects of morphine on hypocretin cells in mice

To determine if the differences in the number and size of hypocretin cell somas seen between human heroin addicts and controls might be induced by opiates, we administered morphine at several doses for varying time periods. Subcutaneous injection of fixed (FD) and escalating doses (ED) of morphine for 7 days in mice did not significantly change the number of detected hypocretin cells. However, 14 day administration of either dose schedule significantly increased hypocretin cell number relative to saline (S) injection (Fig 2a). Fixed doses of 10 mg/kg or larger, for a single 2 week period, significantly increased cell number (Fig 2b), with a maximal average increase of 38% at 50 mg/kg. With daily dosing for a 60 day period, the magnitude of changes in cell number was smaller than that after 14 days of administration (Fig 2c). The effect on hypocretin cell number was largest in the lateral hypothalamus, but a significant increase in

hypocretin cell number was also seen in the medial hypothalamus (Fig 2d). After 14 days of administration of 50 mg/kg of morphine, hypocretin cell number remained significantly elevated for an additional 4 weeks (Fig 2e). After 60 days of administration of the same dose, the significant elevation of hypocretin number lasted 2 weeks. Fig 2f shows the time course of recovery from hypocretin cell shrinkage after morphine administration for 2 weeks. All morphine injections produced waking with activity for ≥ 3 hours. Hypocretin cell size returned to the size seen in saline treated animals by 2 weeks after the last administration (Fig 2f). The increased numbers of cells were within the regions containing hypocretin cells under baseline conditions (Fig 2g). There was no significant change in the percentage of Narp double labelling in the increased number of hypocretin cells in mice treated with 50 mg/kg of morphine for 14 days (Fig 2h). Because subcutaneous implantation of morphine pellets is commonly used in opiate research, we studied the effect of implantation of morphine pellets (25 mg) or control pellets. The slow uptake of the morphine in the pellets maintains an elevated level of the drug, in contrast to the phasic effects of injections. Fig 2i shows the result of the subcutaneous presence of morphine pellets, compared to control pellets, on the distribution of hypocretin cell sizes after 72 hours. On average, cell size was decreased by 23%. So, like morphine injection, morphine pellet implantation shrinks hypocretin cell size, shifting the entire population distribution downwards. However, the continuous presence of morphine for 7 or 14 days, produced by replacing pellets every 3 days, did not produce a significant increase in hypocretin cell number (Fig 2j). MCH cell number in mice was not affected by injection of morphine at 50 or 100 mg/kg for 14 days (Fig 2k). There was also no change in MCH cell size after 14 days of morphine administration (Fig 2l).

Effect of morphine on Narp, dynorphin and MCH levels in mice

A 100 mg/kg escalating dose of morphine (Fig. 3 a-c) was given for 14 days. PCR was performed to assay mRNA levels (a-c). Western blots were used to assess peptide levels (d-e).

The mRNA levels of preprohypocretin, Narp and preprodynorphin, all found in hypocretin neurons, were significantly elevated. Western blot assay showed that 14 day administration of morphine increased preprohypocretin levels by 79% which returned to baseline levels within 2 weeks of withdrawal (WD) (Fig. 3d). Fig 3e shows that there was no significant change in preproMCH levels in the same animals.

Change in hypocretin cell number with morphine is not due to artifact or neurogenesis

Morphine administration at 100 mg/kg for 14 days to wild type (WT) mice increases the number of detected hypocretin cells (Fig 2). But morphine administration to hypocretin knockout (KO) mice does not produce any immunohistochemical detection of hypocretin cells, indicating that labelling requires the presence of hypocretin in neurons (Fig 4a). Fig 4b left, shows no significant change in the number of 5-bromo-deoxyuridine (BrdU) labelled cells throughout the hypocretin cell region after morphine, indicating that the increased number of hypocretin neurons is not due to neurogenesis (photomicrographs in Fig 4b right). Fourteen days of 100 mg/kg morphine treatment also did not produce any change in doublecortin staining of neurons in the hypothalamus, indicative of immature neurons, compared to saline. Doublecortin staining validity is indicated by its normal presence in dentate gyrus (DG) (Fig 4c, right) and the relative lack of doublecortin staining in the hypothalamus of the same animals (Fig 4c left).

Effect of systemic morphine injections on hypocretin unit activity *in vivo* in the rat

A **s**pecies-appropriate dose of 15 mg/kg morphine (higher doses are lethal) produced a greatly elevated discharge rate in hypocretin neurons, accompanied by increased EEG activation and increased electromyographic (EMG) activity in the freely moving rat (Fig 5). Hypocretin neurons were identified using our previously published criteria[9].

Reversal of hypocretin cell decrease in narcolepsy with morphine administration

We used a newly developed transgenic mouse model in which hypocretin cell loss is triggered by doxycycline withdrawal Fig 6 [10]. This model allows induction of selective hypocretin cell loss and symptoms of narcolepsy with cataplexy in mature mice, resembling the pattern that occurs in human narcolepsy. Resuming doxycycline administration stops further hypocretin cell loss in these orexin-tTA;TetO DTA mice ("DTA mice"). Littermate controls were used. The horizontal red line in Fig 6a shows the number of hypocretin cells in control DTA mice maintained throughout on doxycycline, with 14 days of daily saline injection and then sacrificed. The green bar shows littermate mice with doxycycline withdrawal for 1.5 days followed by restoring doxycycline administration and saline injections for 14 days. A 30% reduction of hypocretin cells relative to the control level was seen. But when daily 100 mg/kg morphine injections were given instead of saline for 14 days in a third group, the number of detected hypocretin cells was restored to baseline level (blue bar). Fig 6b shows the effect of morphine administration on cataplexy in two groups of five hypocretin depleted DTA mice. These mice were taken off doxycycline for 18 days, a duration that produces a 95% depletion of hypocretin neurons and cataplexy. Morphine at 50mg/kg greatly reduced cataplexy relative to controls, measured after 1 and 2 weeks of morphine administration [$F(1,6)=148.4$, $p=0.0001$.] (Hypocretin knock out mice, in which the "hypocretin" cells are present but the peptide is absent from birth, show much lower levels of cataplexy than DTA mice[7]. Therefore the response of cataplexy to administration of morphine cannot be easily tested in the knock outs. The extensive loss of most of the hypocretin cell bodies in the DTA mice more closely resembles the anatomy and symptoms of human narcolepsy than the hypocretin knockout mice[7, 10]).

Fig 6c shows control data from human subjects without narcolepsy (green) and data from two narcoleptics. Patient NBB-01064, and patient NBB-08023, were both diagnosed as having narcolepsy with cataplexy (blue bars). Patient NBB-01064 was chronically treated with morphine for relief of her pain resulting from discopathy after her initial narcolepsy diagnosis.

Patient NBB-08023 was not treated with opiates. Eight years later patient NBB-01064 was reclassified as having idiopathic hypersomnia without cataplexy, a very unusual clinical course, suggesting that the extended period of morphine administration may have changed the trajectory of the disease. The view to the right and above, show an expanded view of hypocretin cell counts in the diagnosed narcoleptic with cataplexy without morphine treatment (NBB-08023) and in the morphine treated narcoleptic (NBB-01064). The patient that received morphine at a schedule optimized for pain relief for a 9 year period, had far more hypocretin cells than the untreated narcolepsy patient The morphine treated patient (NBB-01064) had 16% of the control number of hypocretin cells, vs. 3% of the control number in the non-morphine treated narcoleptic with cataplexy patient (NBB-08023). It appears possible that the relatively high number of hypocretin cells was due to the period of morphine administration, as would be predicted from the human heroin addict brains shown in Fig 1 and from the mouse morphine experiments shown in Figs 2 and 6a, although this data by itself is clearly not definitive. These studies were on 6µ paraffin sectioned brains and used different antibodies and counting procedures than prior studies on 40µ frozen sections using stereology (see Supplementary Methods). The optimal morphine dose and administration schedule that might increase the number of neurons producing hypocretin in human narcoleptics remains to be determined. If more brains of narcoleptics treated with opiates become available it may be possible to more precisely describe the relation between morphine dose and administration schedule on hypocretin cell number and symptomatology in human narcoleptics. However, acquiring a sufficient number of such brains would be difficult. In contrast, further work on human opiate addicts may better define the dynamics of the response to opiate self-administration.

DISCUSSION

Cells of different neurotransmitter phenotypes have been characterized by various techniques since the early work by Falck and Hillarp. Most modern research uses immunohistochemistry, the most sensitive technique, to identify and quantify these cell types in normal and pathological human conditions. Hypocretin cell counts determined by immunohistochemistry[1] exceed those determined by genetic labeling and RNA labeling A relevant comparison is between our initial finding of an average of 71,000 hypocretin cells in human controls with a 90% loss in narcolepsy with cataplexy, using immunohistochemistry [1, 11], and a contemporaneous report of 15-20,000 cells in normal controls and no detected hypocretin cells in narcoleptics with cataplexy, using in situ labelling [12]. Independent studies have confirmed the immunohistochemistry work, finding surviving hypocretin cell in all human narcoleptics[13] as we now also show in figure 6c.

Immunohistochemistry can be combined with immediate early gene staining[7] or with juxtacellular labeling and unit recording[9] to characterize the activity of identified cells in normal animals. Immunohistochemistry is also used to identify and study neuropathologies, including cell loss in narcolepsy, Huntington's, Parkinson's, Alzheimer's and other disorders. Our findings here suggest that this approach may be missing an important aspect of brain function. Clearly, as seen in the present study, under some drug administration or disease conditions the number of cells of a particular phenotype visualized with immunohistochemistry can change. A decrease in number of cells can mistakenly be seen as representing neuronal death. But increases of the sort we report here and that we and others have reported previously[14, 15], suggest that some very substantial portion of phenotypically identifiable cells do not produce immunohistochemically detectable levels of their transmitter under most conditions, but can be induced to produce such levels by drugs or by disease. In recent work we have shown that raising hypocretin content by inhibiting microtubule polymerization with colchicine increases the

number of detected hypocretin cells by as much as 40% in wild type mice[16]. This raises the question of the extent to which the reduced number of hypocretin cells that characterizes human narcolepsy and other disorders may, at least partially, be due to reduced production of hypocretin in a subpopulation of hypocretin cells rather than being completely due to neuronal death.

In mice given colchicine, the size of the hypocretin cells increases, possibly as a result of the blockage of axonal transport (16). One may speculate that the greatly increased activity that we see in hypocretin neurons with morphine may have the opposite effect on cell size as peptides/proteins including hypocretin, Narp and dynorphin are transported out of the neurons and down their axons faster than they can be synthesized and ionic pumps work to restore membrane polarization, thereby depleting the substrates for supporting these functions and shrinking the cells. We do not yet know how the peptide production of individual neurons is controlled, how neurons not producing detectable levels of hypocretin differ from those producing detectable levels and how hypocretin expression varies across disorders. Our work reported here shows that the additionally recruited neurons are not uniformly distributed across the population of hypocretin cells, suggesting that they may receive a different pattern of inputs, may project to a different pattern of sites and may differ in other ways from the populations observed prior to opiate administration.

We show that the increased number of hypocretin neurons caused by morphine is not due to neurogenesis. We also demonstrate that the increase lasted well beyond the period when opiates would be detectable in the body's tissues. In mice, the time course of hypocretin cell number increase and cell size decrease differed. The increase required at least 2 weeks of high dose administration and returned to baseline by 8 weeks post administration. However, the cell size reduction occurred within as little as 72 hours of exposure, with size returning to baseline by 4 weeks. It remains to be determined what the time course of such changes is in humans.

But our data indicates that despite varying and indeterminate levels of heroin administration, the effect on hypocretin cell number and size in human addicts has a magnitude equal to or greater than that seen in mice with 14-60 day periods of daily administration. Forty to fifty percent of potential “hypocretin cells,” are apparently not detectable in humans under baseline conditions.

Morphine decreases the size of VTA neurons in rodents[17]. Although, to our knowledge, no major increase (or decrease) in cell number of any group has been previously reported with heroin or morphine administration, opiates have been shown to produce changes in dendritic field and spine morphology in the ventral tegmental field and nucleus accumbens, thought to be the result of altered release of dopamine from VTA cells[18].

We[7] and others[5] have demonstrated that increased hypocretin cell activity is linked to pleasurable but not to aversive tasks in mice and rats. We found that hypocretin is released in non-addict humans when they were engaged in enjoyable tasks, but not when they are aroused by pain or were feeling sad[6]. Conversely, human narcoleptics, who have, on average, a 90% loss of detectable hypocretin cells[1], have greatly elevated levels of depression and are relatively resistant to drug addiction[19]. In opiate addicts, elevating hypocretin production for long periods by self-administration may create a positive affect, and a more negative affect is likely with withdrawal. This feedback loop may contribute to, or underlie addiction.

Dopamine neurons, particularly those located in the ventral tegmental area (VTA) have been strongly implicated in reinforcement in general and addiction in particular. Hypocretin and dopamine are evolutionarily linked from both a neurochemical and anatomical perspective[20]. The VTA receives a major hypocretin projection and projects strongly to the nucleus accumbens. The levels of dopamine and its major metabolites in the nucleus accumbens are markedly increased by the microinjection of hypocretin-1 and hypocretin-2 into the VTA. An intra-VTA injection of a selective hypocretin receptor-1 antagonist, SB334867A, suppresses

morphine-induced place preference. Dopaminergic activation of neurons in the accumbens shell by morphine withdrawal requires the integrity of hypocretin receptor-1[21]. The evidence described above suggests that the hypocretin system may independently mediate some portion of morphine's reinforcing properties.

An *in vitro* slice study found that opioids decrease the activity of hypocretin neurons and that blockade of μ-opioid receptors enhances the activity of hypocretin neurons. Morphine pretreatment inhibited subsequent excitatory responses to hypocretin in hypocretin neurons recorded *in vitro*[18]. However, our current *in vivo* data suggest that systemic administration of morphine greatly increases hypocretin unit activity in rats, an effect presumably mediated at the circuit level, and therefore not seen in the slice. Activation of hypocretin neurons reinstates an extinguished preference for morphine[22].

The VTA, nucleus accumbens, amygdala, locus coeruleus and central gray all have been implicated in reward mediation[23]. Hypocretin cells also contain and release glutamate[24], trigger glutamate release from adjacent cells and contain neuronal activity regulated pentraxin (Narp), an immediate early gene involved in aggregating AMPA receptors and thought to have a role in addiction[8]. Bingham et al.[25] found that hypocretin, like morphine, produces profound analgesia. Aston-Jones et al. showed that hypocretin was required for learning a morphine conditioned place preference task[26]. Georgescu et al.[27] showed that hypocretin neurons, but not nearby MCH neurons, have μ-opioid receptors. cAMP response element-mediated transcription is induced in a subset of hypocretin cells, but not in MCH cells, after chronic exposure to morphine or induction of withdrawal. Additionally, c-Fos and the preprohypocretin gene are induced in hypocretin cells during morphine withdrawal. Constitutive hypocretin KO mice developed attenuated morphine dependence, indicated by a less severe antagonist-precipitated withdrawal syndrome[21].

It has long been anecdotally noted that narcoleptics, who have a 90% loss of hypocretin neurons[1], show little, if any, evidence of drug abuse or addiction, despite their daily prescribed use of gamma hydroxybutyrate (GHB), methylphenidate and amphetamines, drugs that are frequently abused. This data is consistent with our current finding of increased hypocretin cell populations in human heroin addicts, perhaps facilitating and maintaining addiction.

In 1981 a report of a narcoleptic given codeine (a natural isomer of methylated morphine) for the control of Crohn's disease symptoms reported a "disappearance of his narcolepsy, cataplexy, sleep paralysis, and hypnagogic hallucinations[28]" In a second case report, a narcoleptic who could not continue taking stimulant drugs because of coronary artery disease and the necessity for kidney dialysis, urged his doctor to prescribe codeine for his narcolepsy because of the reversal of narcoleptic symptoms he had previously experienced when given codeine for pain. His physician published the results indicating a "dramatic improvement in alertness and substantial reduction of cataplexy", the defining symptoms of narcolepsy[29]. A third paper[30] tested codeine on 27 narcoleptic patients. Sleep diaries and patient reports revealed consistent symptom improvement compared to placebo, however there were no significant differences in the multiple sleep latency test (cataplexy was not tested for). This ambiguous result from a 1 week trial in humans appears to have ended opiate use for treatment of narcolepsy. In human studies, separating the placebo effect from the drug effect can be difficult. We now show, in figure 6b, that opiate treatment is highly effective in reducing or eliminating cataplexy in the narcoleptic mouse. In light of the current findings we are encouraged to think that with the appropriate schedule of administration and dosage, administration of opiate agonists might be an effective treatment for human narcolepsy. Tests to determine the opiate the doses agonists with the least addictive potential and maximal safety and effectiveness are required prior to any recommendation for opiate use in human narcoleptics. An alternate approach might be to develop agonists that more specifically activate hypocretin neurons and thereby increase

hypocretin production without the risks of opiates. The development of appropriate transgenic mice would be an important step in this direction. Until these data are collected it would be inadvisable for narcoleptic patients to self-administer opiates.

Conversely, it appears likely that reducing the number of neurons producing detectable amounts of hypocretin or reducing hypocretin action pharmacologically by opiate receptor antagonists might be a productive approach to the treatment of opiate addiction in humans.

MATERIALS AND METHODS_

Human hypothalamic tissue

The hypothalamus of 5 addicts, 1morphine treated narcoleptic with cataplexy, 1 narcoleptic with cataplexy patient not given morphine and 9 control brains were examined for this study. Characteristics of addicts, narcoleptics and control subjects are summarized in Table I. 5 heroin addict and 7 control brains were fixed in 10% buffered formalin containing 0.1M phosphate buffer (pH 7.4). The hypothalamus was cut into 40 µm sections. Sections were immunostained for hypocretin (Hcrt-1 / orexin-A) and melanin concentrating hormone (MCH). Adjacent sections were Nissl stained. Human nuclear divisions are according Mai et al.[31]. Two narcoleptic and 3 control brains were paraffin embedded. Their sectioning and treatment are explained below.

Hcrt and MCH immunostaining for human addicts and control brains

Hcrt and MCH immunostaining were performed as in our earlier reports[1, 3] The sections were treated with 0.5% sodium borohydride in PBS for 30 min and washed with PBS, and then incubated for 30 min in 0.5% H2O2 for blocking of endogenous peroxidase activity. For antigen retrieval, sections were heated for 30 min at 80°C in a water bath with 10mM sodium citrate (pH 8.5) solution. The sections were cooled to room temperature in sodium citrate and washed with PBS. After thorough washing with PBS the sections were placed for 2 h in 1.5% normal goat serum in PBS and incubated for 72 h at 4°C with a 1:10000 dilution of Hcrt-1 (Rabbit Anti-Orexin A, H-003-30, Phoenix pharmaceuticals Inc., Burlingame, CA, USA). Sections were then incubated in a secondary antibody (biotinylated goat anti-rabbit IgG; Vector Laboratories, Burlingame, CA) followed by avidin– biotin peroxidase (ABC Elite Kit; Vector laboratories), for 2 h each at room temperature. The tissue-bound peroxidase was visualized by a diaminobenzidine reaction (Vector laboratories). Adjacent series of sections were immunostained for MCH (with a 1:20 000, polyclonal rabbit anti-melanin concentrating hormone,

H-070-47, Phoenix Pharmaceuticals Inc., Belmont, CA, USA). In all cases the sectioning and staining were done blind to condition with the same antibody lots used for all subgroups in each study.

Double labeling of Hypocretin and Narp

After blocking using 3% normal donkey serum and 0.3% TTX, sections were incubated with hypocretin antibody (Orexin 1:1000, anti-goat, 8070, Santa Cruz, USA) and Narp (1:1000, anti-rabbit, Worley Lab, John Hopkins, USA) for 72 hrs. The secondary antibodies, 1:400, Alexa Fluor® 488, anti-goat, 1:400 Alexa Fluor® 568, anti-rabbit (Invitrogen, Life Technologists Corporation, USA) were used.

Quantitative analysis (frozen tissue)

Hcrt and MCH cell number, distribution and size were determined in humans with stereological techniques on a one in twelve series of 40µ frozen sections through the complete hypothalamus. We employed a Nikon E600 microscope with three axis motorized stage, video camera, Neurolucida interface and Stereoinvestigator software (MicroBrightfield Corp., Colchester, Vermont). Quantification of Hcrt and Narp double labelling was done using Zeiss Axio Imager M2. In human subjects, the complete hypothalamic region of one half of the human brain was cut into 40 µm thick coronal sections with one in six section interval. One series of sections were stained with cresyl violet for the localization of anatomical regions. Adjacent series of sections were immunohistochemically stained for Hypocretin. After staining, the sections were serially arranged and mounted on slides. Hcrt cells were individually counted with Neurolucida program in each section. The final number reported is the number for the whole brain based on our systematic count. Sectioning, staining and counting were done by investigators blind to condition. In our initial human studies (1, 3) we confirmed the results of stereological sampling with exhaustive counting of hypocretin neurons. In mice we completely

counted, bilaterally the number of hypocretin neurons on a 1 in 3 series and report the resulting number without multiplying by 3.

Hcrt immunostaining for narcoleptic and control brains (paraffin fixed tissue)

Sections were incubated with rabbit anti-hypocretin A antibody (cat. No H-003-30, lot no 01169-4, Phoenix pharmaceuticals, Inc., Burlingame, CA, USA) at 1:20000 diluted in TBS-milk (5% milk w/v in 0.05 M Tris,0.15 M NaCl, pH 7.6) for an hour at room temperature, followed by incubation overnight at 4°C. The next day, after rinsing in TBS, sections were incubated with goat-anti-rabbit serum at 1:400 in TBS for 1 hour at room temperature. Antibody binding was visualized according to the ABC method at a 1:800 dilution of these complexes in TBS for 1 hour at room temperature. After rinsing in TBS, staining was developed by DAB, nickel-ammonium sulfate for approximately 20 min. Reactions were stopped by washing sections in distilled water. Finally, slides were dehydrated in an ascending series of alcohol and coverslipped in xylene with Entellan.

Counting procedure for paraffin sections

Only positively stained neurons containing a nucleolus were included in order to prevent double counting. This counting procedure, which was judged to be the best for the thin (6 µm) sections used, is based on the principle that nucleoli can be considered as hard particles that will not be sectioned by a microtome knife but, instead, are pushed either in or out of the paraffin when hit by the knife[32]. All the cell counts were from one side of hypothalamus. Completeness of the cell counting was confirmed by graphically presenting the actual number of neurons counted in every section from rostral to caudal to review the distribution pattern. If the most rostral or caudal sections still showed positive cells, we cut the remaining tissue so as to have a complete series.

Following hypocretin staining, the total number of neurons was estimated at 600 µm intervals throughout the area. In each section, all hypocretin neurons with their typical cell profiles and a visible nucleolus serving as a unique marker for each neuron, were counted using light microscopy at a magnification of 400x. Taking into account the interval distances between individual sections the total number of hypocretin neurons was determined based on the Cavalieri principle[33].

Animal Study

All procedures were approved by the Institutional Animal Care and Use Committee of the University of California at Los Angele (UCLA) and the Veterans Administration Greater Los Angeles Health Care System (VAGLAHS).

Morphine pellet study in mice

Experiments were performed on male C57BL/6 mice weighing 25 - 30 g. Animals were housed on a 12-h light–dark cycle. Food and water were available ad libitum. The characteristics in each group of the study are detailed in Table II. 25 mg morphine pellets (from NIDA) and placebo pellets (NIDA) were subcutaneously implanted with halothane anesthesia. There were three groups for the pellet study (1) pellet implanted for 3 days, (2) pellet implanted for 7 days and (3) pellet implanted for 14 days. For groups 2 & 3 the initial pellet was replaced after 72 hours. All animals were killed between 12.00-14.00 h. Animals were anesthetized with Fatal - Plus solution (i.p.), then perfused transcardially with PBS followed by 4% paraformaldehyde in PBS. Brains were removed and post-fixed for 72 h in 4% paraformaldehyde in PBS followed by 30% sucrose in PBS. The sections were cut at 40 µm on a sliding microtome and stained for Hcrt as described earlier. Mouse nuclear divisions are as in McGregor et al., 2014[7].

Morphine dose response in mice

A 14 day dose response trial with daily administration of a fixed dose of morphine (Morphine sulfate, Hospira Inc., Lake Forest, IL, USA) in each group of animals from 1 to 100 mg/kg body weight were conducted. Morphine dissolved in sterile saline was injected subcutaneously. The doses were 1 mg/kg, 5 mg/kg, 10 mg/kg, 25 mg/kg, 50 mg/kg, 75 mg/kg and 100mg/kg. Control groups received saline injections. (ii) Morphine escalating dose: This started with 100mg/kg and was increased by 20% each 72 hours. (iii) 60 day dose response: There were three doses, 10 mg/kg, 25 mg/kg and 50 mg/kg body weight. Control groups received saline. Injections were done at 10:00. All animals were killed between 12:00 and 14:00 h.

Morphine 50 mg/kg for 14 days and withdrawal for up to 6 months

To study the effect of morphine withdrawal on the Hcrt system we administered 50 mg/kg for 14 days. The control group received saline.

Morphine 50 mg/kg for 60 days and withdrawal up to 6 months

To study the effect of long duration administration of morphine, we gave 50 mg/kg for 60 days. The control groups received saline. (1) 2 week withdrawal, (2) 4 week withdrawal, (3) 8 week withdrawal, (4) 16 week withdrawal and (5) 26 week withdrawal durations were employed.

Investigation of neurogenesis after morphine treatment in mice

To look for evidence of neurogenesis after morphine treatment 5-Bromo-2-Deoxyuridine (BrdU) was given intraperitoneally at 50 mg/ kg in sterile saline once daily for two weeks. BrdU injection was done in morphine treated (100 mg/kg) and saline treated animals. Morphine injection was done in the morning and BrdU injection was done in the evening for the two week period. Animals were sacrificed two weeks after the initial injection or 4 weeks after the end of two weeks of injection period. There were three animals in each experimental group.

Immunohistochemistry

Brains were sectioned at 40 µm in the frontal plane through the hypothalamus. Immunohistochemistry for BrdU was performed on every fourth, free-floating section. Tissue was pretreated for BrdU immunostaining by DNA denaturation (2M HCl at 37°C for 30 min) followed by 10 min in borate buffer (pH 8.5). Sections were then incubated with rat anti-BrdU monoclonal antibody (1:400; Novus Biologicals, USA) for 72 hrs. Sections were developed using the ABC and DAB methods (Vector Elite). Doublecortin staining was done using goat anti-doublecortin C-18 (DCX 1:1000, # SC-8066, Santa Cruz Biotechnology, USA).
Morphine and Hcrt -/- mice: Homozygous male Hcrt -/- mice were injected 100 mg/kg morphine subcutaneously. The control group received saline (n = 3 each group, Table II). The animals were sacrificed on 14th day and Hcrt immunohistochemistry performed.

qPCR procedure

Morphine escalating dose started from 100mg/kg and was escalated by 20% every 72 h ending up at 180 mg/kg for two weeks. Brain samples from mice (8 Saline injected mice vs. 8 morphine injected mice) were snap frozen by dry-ice and stored in -80°C. They were cut into 200 µm sections in a cryostat at -18 °C. The hypothalamus was bilaterally punched out by pre-chilled 1.0 mm punching needle (Miltex, Inc. York, PA, USA). Brain tissue samples were pooled with two animals in each tube and immediately put back on dry ice. The tissue was homogenized with 1000 µl QIAzol Lysis Reagent (QIAGEN Sciences. Maryland, USA) and 200 µl chloroform (Merck, Darmstadt, Germany). RNA was isolated according to the RNeasy Lipid Tissue Mini Kit (Qiagne Cat. No. 74804) manufacturer's protocol. RNA concentration and quality was measured by a Nanodrop TM ND-1000 spectrophotometer (Thermo Fisher Scientific. MA, USA). For each sample, 1000 ng of total RNA was used for synthesis of cDNA, as described by the manufacturer's protocol of the iScript cDNA synthesis Kit (Bio-RAD Lab. Hercules. CA, USA).

Primer sequences for PreproHypocretin/orexin, Preprodyn and Narp GenBank accession code are indicated below. Primer sequences for glyceraldehydes-3-phosphate dehydrogenase (GAPDH), Tubulin, alpha 1A (Tuba1a), Ribosomal protein S28 (28s), Actin-beta, ubiquitin C (UBC), Tubulin, beta 4a (Tubb4a) were used as reference genes.

The Quantitative PCR (qPCR) procedures have been described previously[34]. In short qPCR was performed in a reaction volume of 20 µl, using the SYBR Green PCR kit (Promega, Madison, WI, USA) and a mixture of sense and antisense primers (2 pmol/µl). Reactions were run in a GeneAmp 7300 thermocycler under the following conditions: 2 min at 50^{o}C and 10 min at 95^{o}C, followed by 40 cycles of 15 sec at 95^{o}C and finally 1 min at 60^{o}C. Data were acquired and processed automatically by the Applied Biosystems Sequence Detection Software. Specificity of amplification was checked by melting curve analysis and electrophoresis of products on 1.5% Agarose gel. Sterile water (non-template control) and omission of reverse transcriptase (non-RT control) during cDNA synthesis served as negative controls.

Amplification efficiency was determined by running qPCR reactions on a dilution series of pooled cDNA from all the subjects. Resulting cycle threshold (Ct) values were plotted against the inverse log of each dilution and the slope of this curve was then used to calculate the efficiency as follows: Efficiency (E) = 10- (1/slope). (below) The normalization factor was based upon the geometric mean of the following 2 most stable reference genes (i.e. Actin-β and Tubb4a) out of 6 candidate reference genes selected by geNorm analysis[35]. To minimize handling variations, each gene was measured in triplicate. The relative absolute amount of target genes calculated[36] was divided by the normalization factor. Note: 28S= Ribosomal protein S28; Actb=Actin, beta; GADPH1= Glyceraldehyde-3-phosphate dehydrogenase; Pdyn= prodynorphin; PHcrt= Prepro-hypocretin/orexin; Tuba1a= Tubulin, alpha 1A; Tubb4a= Tubulin, beta 4A class Iva; NARP= neuronal pentraxin 2 (Nptx2); Ubc= Ubiquitin C.

Western blots- PPO and pro-MCH

The mouse hypothalamus was sonicated in lysis buffer containing 50 mM Tris HCl, 50mM MgCl2, 5mM EDTA and a protease inhibitor tablet (Roche, cat# 12482000) and centrifuged at 800xg (3,000 rpm) for 30 min. at 4°C. Protein concentration of the supernatant was determined using the DC Protein assay kit (BioRad, cat# 500-0112). Forty µg of protein was loaded on a 12% mini-protean TGX precast gel (BioRad, cat# 456-1044) and separated at 50V. The proteins were then transferred to a PVDF membrane (BioRad, cat#, 162-0176) at 50 mA for 2h. The membrane was washed in 20mM Tris, 150mM NaCl, 0.1% Tween 20 (TBST) and then blocked in TBST containing 5% (w/v) nonfat dry milk (NFDM) for 1h. The membrane was incubated with rabbit anti-PPO (Santa Cruz, sc-28935) and rabbit anti- MCH (Santa Cruz, sc-28931) at 1:1,000 dilution in 2.5% NFDM in TBST overnight at 4°C. The next day the membrane was washed in TBST before incubation with goat anti-rabbit HRP conjugated secondary antibody (Thermo Scientific, cat # 31463) at a dilution of 1:10,000 in 2.5% NFDM in TBST, for 1 h at room temperature. After washing in TBST, the antibody complex was visualized with SuperSignal West Femto (Thermo Scientific cat# 34094). Anti- ß-actin (Millipore MAB1501R) was used as an internal normalizer, at a dilution of 1:10,000, with goat anti-mouse HRP conjugated secondary antibody (Sigma, cat # A2304) at a dilution of 1:10,000. The densities of PPO, pro-MCH and ß-actin bands for each sample were measured using Image J software.

Morphine and DTA mice

To create a model of orexin/hypocretin deficiency with closer fidelity to human narcolepsy, diphtheria toxin A (DTA) was expressed in orexin neurons under control of the Tet-off system[10]. Male orexin-tTA; TetO DTA mice were maintained from weaning to 10 weeks of age on DOX (+) chow. To reduce the number of orexin neurons without elimination of the entire cell population, DOX (+) chow was removed at 10 weeks of age and replaced with Labo MR stock food (DOX

(−) condition) for 1.5 d, after which DOX (+) chow was reintroduced (1.5 d + restoration of DOX; n = 8). The experimental group (n = 4) then received 100mg/kg morphine subcutaneously for 14 days and the control group received (n = 4) saline. Two hours after last day injections, mice were deeply anesthetized with pentobarbital (50 mg/kg, i.p.) and perfused sequentially with 15 ml of chilled saline and 15 ml of chilled 10% formalin solution (Wako). Brains were isolated and immersed in formalin solution for 72 h at 4°C, followed by 30% sucrose. The sections were cut at 40 µm and stained for Hcrt as described earlier.

Cataplexy scoring in DTA mice treated with morphine

Female Orexin tTA x tetO DTA mice (Inutsuka et al., 2012), aged 4-6 months fed doxycycline food from birth (as were their mothers prior to the birth of the pups), were placed on regular laboratory rodent food for 18d and then back on doxycycline food (Teklad cat# TD.130840) for 1-2 months. The mice were singly housed (lights on at 6 AM and off at 6 PM). The mice were recorded from 5PM-6AM the next day, via a LOREX DV700 recording system with a 1080p HD MPX DVR. Chocolate (“Hershey’s kisses”, milk chocolate) was given at 6PM to enhance cataplexy attacks (Tabuchi et al., 2014). This was recorded as baseline cataplexy. At 10AM, the mice were injected subcutaneously with either saline or 50 mg/Kg morphine. Morphine or saline was administered every day at 10AM, for 2 weeks. Overnight video recording was repeated weekly, after 1 week and 2 weeks of morphine or saline injections, and for 3 weeks after the termination of morphine/saline treatment. The number of cataplexy bouts was scored for the first 2h (from 6PM-8PM) of the dark phase. Cataplexy was scored based on the criteria of Chemelli et al.[37] and Scammel et al.,[38]: An abrupt episode of nuchal atonia with immobility lasting at least 10 seconds with at least 40 seconds of wakefulness preceding the episode. The number of cataplexy episodes were normalized with each subject’s baseline score and expressed as the percentage of saline control on week 1.

Effect of morphine on Hcrt cell activity in vivo

Male Sprague Dawley Rat (Charles River, Hollister, CA, USA) weighing 250-300g were used (n = 5). Hypocretin cells were recorded from the hypothalamus using microwire recording techniques as described previously[39]. Under isoflurane anesthesia, microdrives containing 25µ stainless steel microwires (California Fine Wire Co., Grover Beach, CA, U.S.A.) aimed at the lateral hypothalamic area were implanted, with the tip of the electrodes 0.5 mm above the target area. Stainless steel screw electrodes were placed over sensorimotor cortex for EEG and electromyogram (EMG) activity was recorded from the dorsal neck muscles with Teflon-coated multistranded stainless steel wires (Cooner Wire, Chatsworth, CA, U.S.A). Animals were free to move around the recording chamber. Electrodes were moved in 80µ steps until a cell or cells with signal to noise ratio of at least 2:1 were isolated. The activity of each cell was then characterized throughout sleep/waking states. Waking-specific cells that fit the profile of Hcrt cell[9] were studied after intraperitoneal injections of morphine (10-15 mg/kg) with continuous recording of neuronal activity for at least 3 hours.

Reference List

1. Thannickal,T.C. *et al.* Reduced number of hypocretin neurons in human narcolepsy. *Neuron.* **27**, 469-474 (2000).

2. Fronczek,R. *et al.* Hypocretin (orexin) loss in Parkinson's disease. *Brain.* **130**, 1577-1585 (2007).

3. Thannickal,T.C., Lai,Y.Y., & Siegel,J.M. Hypocretin (orexin) cell loss in Parkinson's disease. *Brain* **130**, 1586-1595 (2007).

4. C.D.C. www.cdc.gov/drugoverdose/epidemic/. *Centers for Disease Control and Prevention*(2017).

5. Baimel,C. *et al.* Orexin/hypocretin role in reward: implications for opioid and other addictions. *Br. J Pharmacol.* **172**, 334-348 (2015).

6. Blouin,A.M. *et al.* Human hypocretin and melanin-concentrating hormone levels are linked to emotion and social interaction. *Nature Communications* **4:1547**, 1547 (2013).

7. McGregor,R., Wu,M.-F., Barber,G., Ramanathan,L., & Siegel,J.M. Highly specific role of hypocretin (orexin) neurons: differential activation as a function of diurnal phase, operant reinforcement vs. operant avoidance and light level. *Journal of Neuroscience* **31**, 15455-15467 (2011).

8. Blouin,A.M. *et al.* Narp immunostaining of human hypocretin (orexin) neurons: Loss in narcolepsy. *Neurology* **65**, 1189-1192 (2005).

9. Mileykovskiy,B.Y., Kiyashchenko,L.I., & Siegel,J.M. Behavioral correlates of activity in identified hypocretin/orexin neurons. *Neuron* **46**, 787-798 (2005).

10. Tabuchi,S. *et al.* Conditional Ablation of Orexin/Hypocretin Neurons: A new mouse model for the study of narcolepsy and orexin system function. *J Neurosci* **34**, 6495-6509 (2014).

11. Thannickal,T.C. *et al.* Human narcolepsy is linked to reduced number, size and synaptic bouton density in hypocretin-2 labeled neurons. *Abstr Soc Neurosci* **26**, 2061 (2000).

12. Peyron,C. *et al.* A mutation in a case of early onset narcolepsy and a generalized absence of hypocretin peptides in human narcoleptic brains. *Nat. Med.* . **6**, 991-997 (2000).

13. Crocker,A. *et al.* Concomitant loss of dynorphin, NARP, and orexin in narcolepsy. *Neurology* **65**, 1184-1188 (2005).

14. John,J. *et al.* Greatly increased numbers of histamine cells in human narcolepsy with cataplexy. *Ann Neurol.* **74**, 786-793 (2013).

15. Valko,P.O. *et al.* Increase of histaminergic tuberomammillary neurons in narcolepsy. *Ann Neurol.* **74**, 794-804 (2013).

16. McGregor,R., Shan,L., Wu,M.-F., & Siegel,J.M. Diurnal fluctuation in the number of hypocretin/orexin and histamine producing: implication for understanding and treating neuronal loss. *PLoS ONE*(2017).

17. Mazei-Robison,M.S. *et al.* Role for mTOR Signaling and Neuronal Activity in Morphine-Induced Adaptations in Ventral Tegmental Area Dopamine Neurons. *Neuron* **72**, 977-990 (2011).

18. Li,Y. & van den Pol,A.N. mu-opioid receptor-mediated depression of the hypothalamic hypocretin/orexin arousal system. *Journal of Neuroscience* **28**, 2814-2819 (2008).

19. Dauvilliers,Y., Lopez,R.+., Ohayon,M., & Bayard,S. Hypersomnia and depressive symptoms: methodological and clinical aspects. *BMC Med* **11**, 78 (2013).

20. Stefano,G.B. & Kream,R.M. Endogenous morphine synthetic pathway preceded and gave rise to catecholamine synthesis in evolution (Review). *Int J Mol. Med.* **20**, 837-841 (2007).

21. Sharf,R., Sarhan,M., & DiLeone,R.J. Orexin mediates the expression of precipitated morphine withdrawal and concurrent activation of the nucleus accumbens shell. *Biol Psychiatry.* **64**, 175-183 (2008).

22. Harris,G.C., Wimmer,M., & Aston-Jones,G. A role for lateral hypothalamic orexin neurons in reward seeking. *Nat.* **437**, 556-559 (2005).

23. Sun,W. Dopamine neurons in the ventral tegmental area: drug-induced synaptic plasticity and its role in relapse to drug-seeking behavior. *Curr Drug Abuse Rev.* **4**, 270-285 (2011).

24. Torrealba,F., Yanagisawa,M., & Saper,C.B. Colocalization of orexin a and glutamate immunoreactivity in axon terminals in the tuberomammillary nucleus in rats. *Neuroscience* **119**, 1033-1044 (2003).

25. Bingham,S. *et al.* Orexin-A, an hypothalamic peptide with analgesic properties. *Pain* **92**, 81-90 (2001).

26. Aston-Jones,G. *et al.* Lateral hypothalamic orexin/hypocretin neurons: A role in reward-seeking and addiction. *Brain Research* **1314**, 74-90 (2010).

27. Georgescu,D. *et al.* Involvement of the lateral hypothalamic peptide orexin in morphine dependence and withdrawal. *J Neurosci* **23**, 3106-3111 (2003).

28. Harper,J.M. Gelineau's narcolepsy relieved by opiates. *Lancet.* **1**, 92 (1981).

29. Benbadis,S.R. Effective treatment of narcolepsy with codeine in a patient receiving hemodialysis. *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy* **16**, 463-465 (1996).

30. Fry,J.M., Pressman,M.R., DiPhillipo,M.A., & Forst-Paulus,M. Treatment of narcolepsy with codeine. *Sleep.* **9**, 269-274 (1986).

31. Mai,J.K., Assheuer,J., & Paxinos,G. *Atlas of the human brain*(Elsevier Academic Press,2004).

32. Fronczek,R. *et al.* Hypocretin (orexin) loss and sleep disturbances in Parkinson's Disease. *Brain.* **131**, e88 (2008).

33. Verwer,R.W. & Raber-Durlacher,J.E. Efficient and unbiased estimation of volume and area of tissue components and cell number in gingival biopsies. *J Periodontal Res.* **28**, 313-323 (1993).

34. Shan,L. *et al.* Alterations in the histaminergic system in the substantia nigra and striatum of Parkinson's patients: a postmortem study. *Neurobiol. Aging.* **33**, 1488-13 (2012).

35. Vandesompele,J. *et al.* Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* **3**, RESEARCH0034 (2002).

36. Kamphuis,W. *et al.* Prostanoid receptor gene expression profile in human trabecular meshwork: a quantitative real-time PCR approach. *Invest Ophthalmol. Vis. Sci.* **42**, 3209-3215 (2001).

37. Chemelli,R.M. *et al.* Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. *Cell* **98**, 437-451 (1999).

38. Scammell,T.E., Willie,J.T., Guilleminault,C., & Siegel,J.M. A consensus definition of cataplexy in mouse models of narcolepsy. *Sleep* **32**, 111-116 (2009).

39. Wu,M.F. *et al*. Locus coeruleus neurons: cessation of activity during cataplexy. *Neuroscience* **91**, 1389-1399 (1999).

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All authors declare no competing financial interest.

Figures

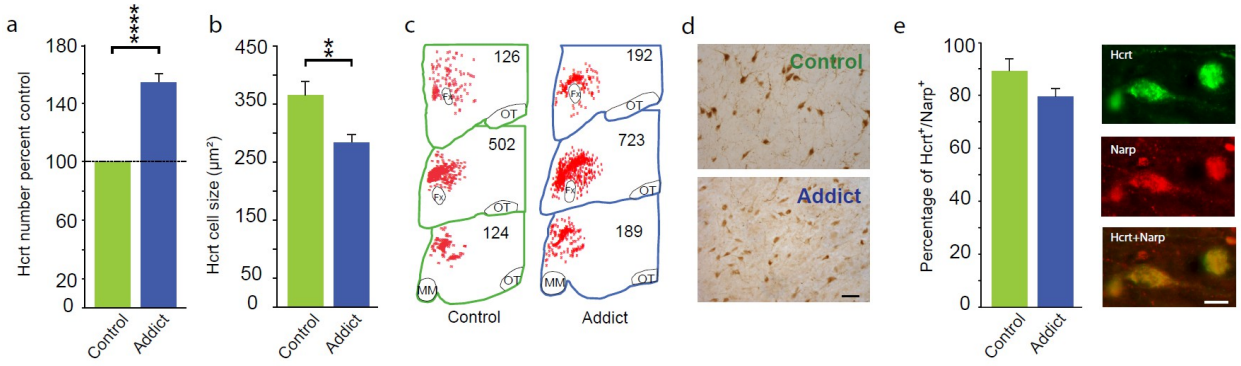


Fig. 1 Human heroin addicts have increased number of hypocretin (Hcrt, orexin) neurons: Subject characteristics are presented in Table 1.

Fig 1a There was a 54% increase in the number of detectable hypocretin neurons in human heroin addicts (N=5) relative to human controls (N=7). This difference was significant (p=0.0009, t=8.89, df=10).

Fig 1b Hypocretin cells were 22% smaller in the addicts (p<0.01, t=2.78 df=10).

Fig 1c Neurolucida mapping illustrates the distribution and increased number of hypocretin cells in addicts relative to controls. Representative counts are given at 3 levels.

Fig 1d Examples of immunohistochemical labelling of hypocretin cells in control and addict. Cal. 50µm.

Fig 1e Approximately 90% of hypocretin neurons in human controls also contain neuronal activity regulated pentraxin (Narp). This percentage does not significantly differ in the larger number of hypocretin neurons observed in human heroin addicts. Fig 1e left shows the mean percentage of hypocretin neurons containing Narp in addicts and controls. Fig 1e right shows hypocretin, Narp and double labelled cells.

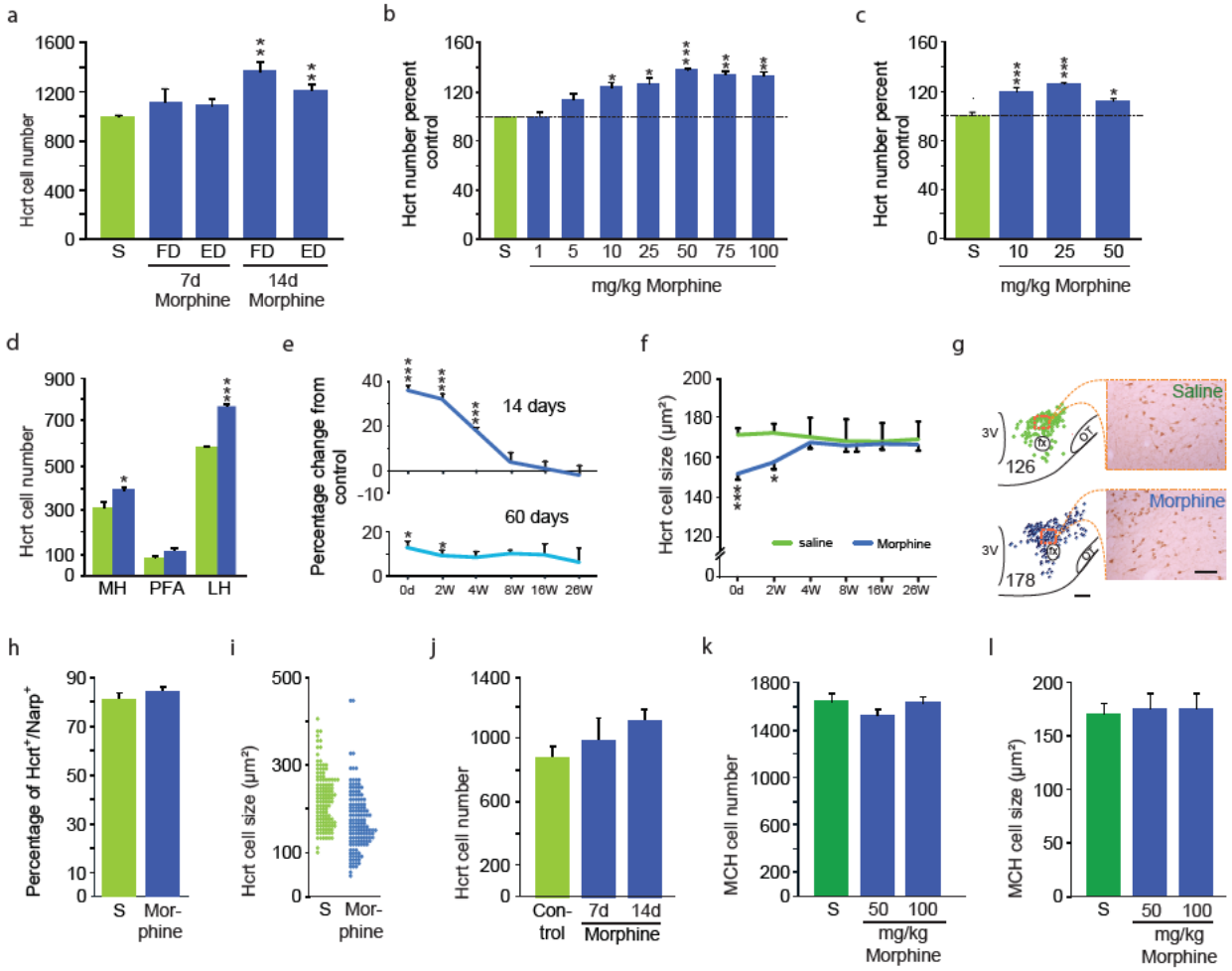


Fig. 2 Dose dependent effects of morphine on hypocretin cells in mice. To determine if the changes in number and size of hypocretin cells seen in human heroin addicts caused, or resulted from, heroin addiction, we administered morphine to mice. [Saline treated animals (S) indicated in green and morphine in blue.] In each case the morphine treated groups were run with a saline treated group of matched age. Details on the subjects and the parameters of treatment are presented in Table 2.

Fig 2a The effect of a fixed dose (FD) of 100 mg/kg for 7 days, or an escalating dose (ED) starting at 100 mg/kg for 3 days and increasing by 20% every 3rd day for 7 days. These doses did not significantly increase hypocretin cell number. However, a fixed dose of 100 mg/kg for 14 days and an escalating dose for 14 days (with a final dose of 180 mg/kg) both increased hypocretin cell number ($p=0.01$, $t=-4.23$ $df=4$; +22%, $p=0.01$, $t=-4.52$ $df=4$ respectively).

Fig 2b A 14 day dose-response trial with daily administration of a fixed dose of from 1 to 100 mg/kg. Doses of 10 mg/kg or higher all produced a significantly elevated number of hypocretin neurons compared to saline. The elevation in hypocretin cell number at 50 mg/kg was 38%. Doses above 50 mg/kg produced no further increase. (ANOVA, normality, variance tests passed between groups, df 7, $F=8.1$, $P<0.001$, 10mg - $p=0.009$, $t=-4.77$ $df=4$; 25mg - $p=0.019$,$t=-3.81$, $df=4$; 50mg - $p=0.002$, $t=-7.07$, $df=4$; 75mg - $p=0.01$, $t=-5.14$, $df=4$; 100mg - $p=0.01$, $t=-4.52$ $df=4$; Bonferroni: 50 <0.001).

Fig 2c The effect of longterm (60 day) daily administration of morphine at doses of 10, 25 or 50 mg/kg. All 3 doses produced significant increases, with the largest increase (+26%) at 25 mg/kg (ANOVA, normality, variance tests passed between groups, df 3, F=8.1, P<0.001, P=0.005, t=-4.41, df=8). Long term morphine dose effect of 10mg/kg for 60 days is +20.21±1.5% (mean ±SEM) p=0.002, t=4.65, df=7). At 50mg/kg 60 days +12.72±2.9%, p=0.031, t=-2.69, df=7; Bonferroni: 10= <0.02, 25 p<0.007, 50 p<0.001, 75p <0.004, 100 <0.004). Thus the optimal dose for increasing hypocretin cell number decreases with this longer period of administration, suggesting adaptation.

Fig 2d The mediolateral distribution of increased hypocretin cell number after 14 days of administration at 50 mg/kg. The effect is largest in lateral hypothalamus (LH) and is also significant in the medial hypothalamus (MH) (LH - p=0.001, t=-11.94, df=7; MH -p=0.05, t=-2.44, df=7).

Fig 2e The duration of hypocretin effects after termination of daily 50 mg/kg morphine administration starting with the day after the final injection (day 0W [W-withdrawal]) relative to control. After 14 days of morphine administration, hypocretin cell number remained significantly elevated for 4 weeks (t=-6.13, df=8, p=0.001, ANOVA, normality, variance tests passed between groups, df 11, F=17.5, p<0.001). After 60 days of administration (bottom), the significant elevation of hypocretin number lasted 2 weeks (t=-4.53, df=8, p=0.004, Bonferroni: 26W p<0.014, ANOVA, normality, variance tests passed between groups, df 5, F=4.9, p<0.001).

Fig 2f A 12.8 ± 2.8 % hypocretin cell size decrease occurred after administration of 50 mg/kg of morphine for 14 days (p=0.001, t=4.88 , df=10). With 4 weeks of withdrawal, hypocretin cell size returned to the size seen in saline treated animals.

Fig 2g Neurolucida plots and photomicrographs illustrating the increased number and the distribution of hypocretin cells after 2 weeks of morphine administration at 50 mg/kg FD. Numbers indicate cell counts in section. Cal. 150µm (on plot) and 50µm (on photomicrographs).

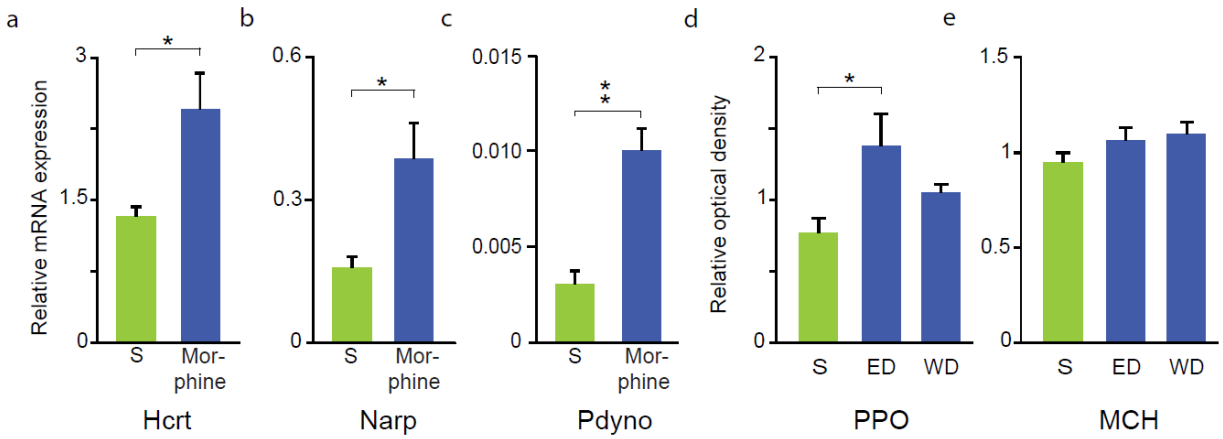
Fig 2h There is no change in the percentage of Narp double labelling in the increased number of hypocretin cells in mice treated with 50 mg/kg of morphine for 14 days compared to controls.

Fig 2i We measured the effect of morphine pellet implantation on hypocretin cell number and size compared to control pellets after 72 hours. On average, cell size was decreased by 23% (P=0.001, Mann-Whitney Rank Sum Test, t=22089.0, n=128 cells in control (green), n=149 cells in morphine (blue). Cell number was unchanged.

Fig 2j The effect of replacement of depleted morphine or control pellets at 3 day intervals for up to 7 or 14 days. These continuous administration paradigms did not produce significant changes in hypocretin cell number at 7 days or 14 days.

Fig 2k MCH cell number is not affected by administration of morphine at 50 or 100 mg/kg for 14 days.

Fig 2l There is also no change in MCH cell size after 14 days of morphine administration. *P<0.05, **P<0.01, ***P<0.001. Opt - optic tract, 3v – third ventricle, W – week.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b
c
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e
Relative mRNA expression
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1.5
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0.6
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S
Mor-
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Narp
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PPO
MCH

Fig.3 Effect of morphine on Narp, dynorphin and MCH in mice. An escalating dose of morphine, starting at 100 mg/kg (see Methods), was given for 14 days and qPCR performed to assay mRNA levels (a-c) and western blots used to assess peptide levels (d, e).

Fig 3a-c mRNA levels of preprohypocretin, Narp and preprodynorphin, all found in hypocretin cells, were significantly elevated. (hypocretin p=0.03, t=2.99 df=5; Narp p=0.02, t=3.36, df=5; Prodynorphin p=0.01, t=3.65 df=5).

Fig 3d Western blot assay showed that 14 day administration of morphine increased preprohypocretin levels by 79% (Fig. 3d, p=0.05, t=-2.51 df=6 in each group) and recovered to baseline within 2 weeks.

Fig 3e There was no significant change in preproMCH levels with morphine administration. This remained nonsignificantly different from control after withdrawal, ED, escalating dose, WD, 2 week withdrawal, *p<0.05, **p<0.01.

Fig. 4 The change in hypocretin cell number with morphine is not due to artifact or neurogenesis.

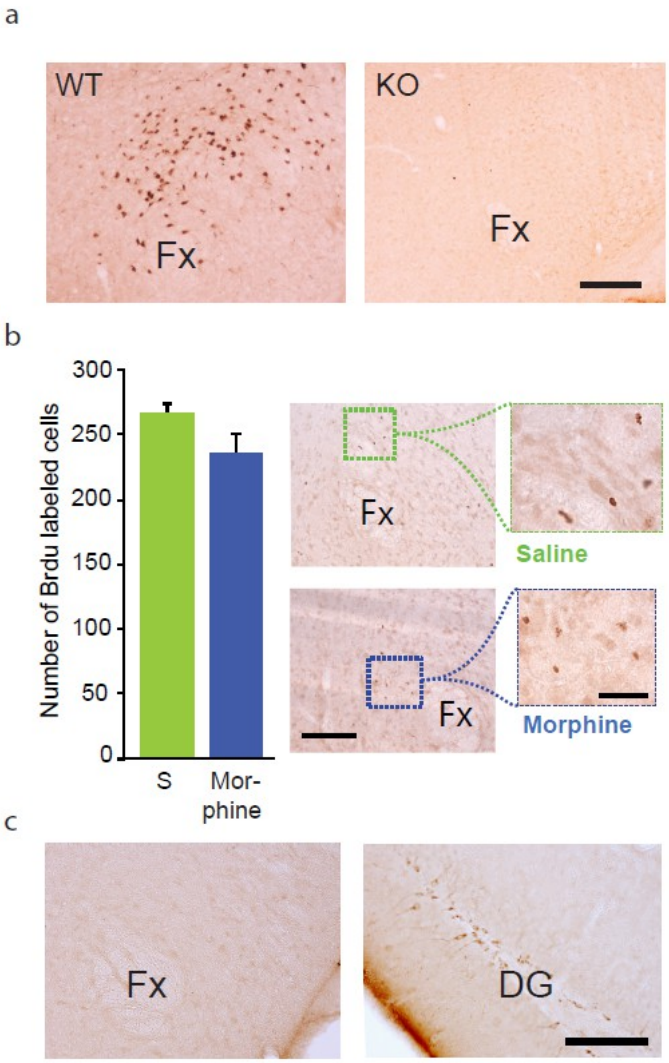

Fig 4a Morphine administration at 100 mg/kg for 14 days produced labeling in WT mice, but did not produce any hypocretin labelling in hypocretin KO mice, indicating that labelling requires the presence of hypocretin in neurons. Cal. 200µm

Fig 4b Increased number of hypocretin neurons was not due to neurogenesis. BrdU labelling to identify new neurons shows no significant increase in the number of BrdU labelled cells in the hypothalamic hypocretin cell field after 14 days of 100 mg/kg morphine treatment of mice, compared to saline (left panel). Right panels show BrdU labelled cells in the perifornical area of saline (top) and morphine (bottom) treated animals. Cal. 100 and 40µm.

Fig 4c Immunohistochemistry for doublecortin. 14 days of 100 mg/kg morphine treatment did not produce any change in doublecortin staining in hypothalamus. Left panel photomicrograph of shows absence of doublecortin staining in the hypothalamus of a treated animal. Right panel photomicrograph shows normal doublecortin staining in dentate gyrus (DG) of the same animal. WT- wild type (C57BL/6 mice), KO - hypocretin -/- mice, Fx - fornix, DG - dentate gyrus. Cal. 100µm.

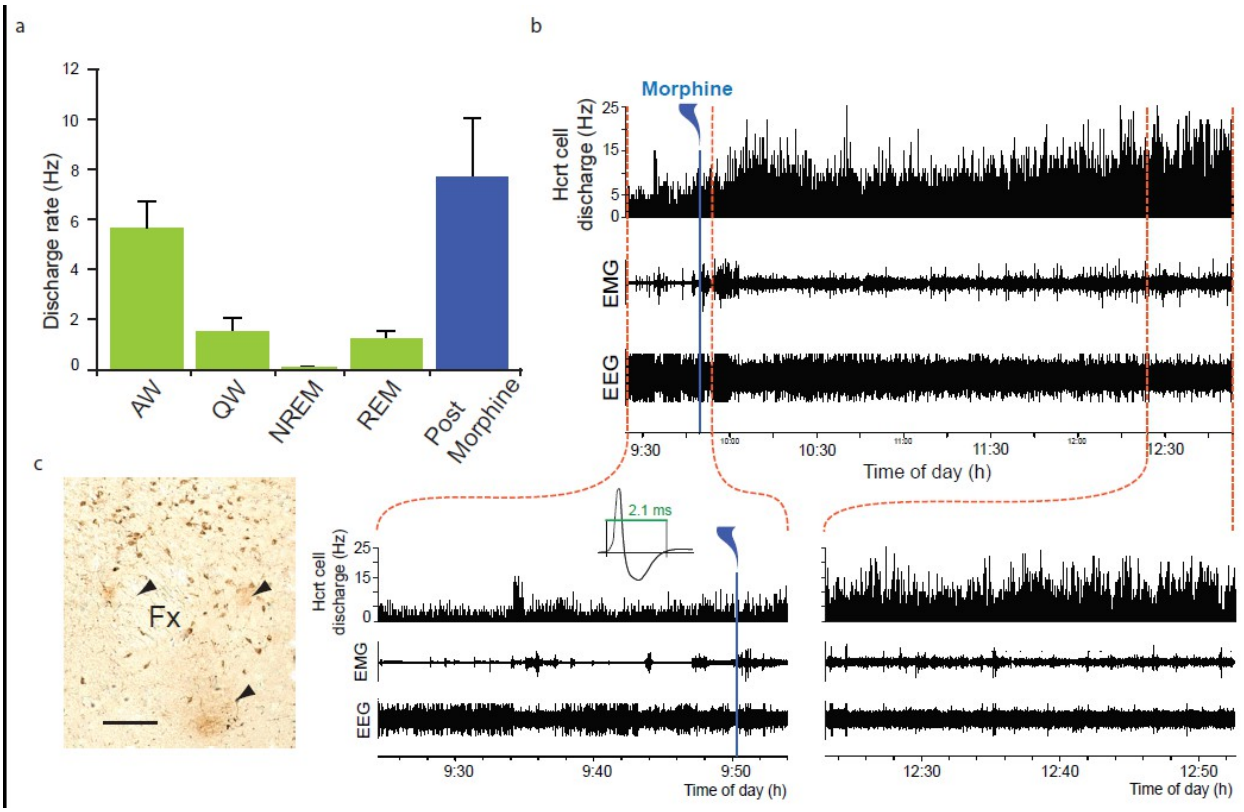

Fig. 5a Effect of morphine on hypocretin cell activity in vivo. The discharge rate of a representative hypocretin neuron after morphine administration of the species appropriate dose of 15 mg/kg of morphine, with expansions below to better show EEG immediately after injection (left) and 3 hours after injection right. The increased discharge rate in hypocretin neurons, along with EEG activation lasted 3 or more hours after injection of morphine, far longer than typical active waking periods. Inset shows average waveform of the hypocretin neuron

Fig 5b Sleep rates are averages of mean rate determined by five 10 sec samples in each of 5 hypocretin neurons, from 3 rats, in each sleep state (AW=active waking; QW=quiet waking; NREM=nonREM sleep, morphine=post injection rate ± SEM). Post-morphine injection rate is based on five, 10 sec samples in each neuron taken 15 minutes after morphine injection. **Fig 5c** Histology showing three recording sites of hypocretin neurons, labelled with arrowheads. Cal. 150µm

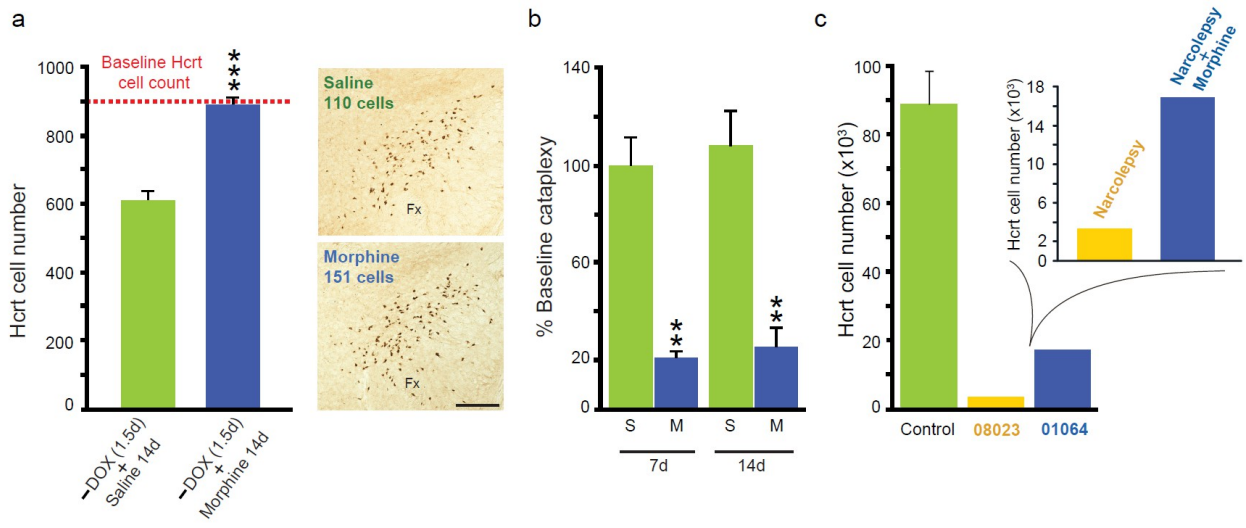

Fig. 6 Reversal of hypocretin cell loss and cataplexy in narcolepsy with morphine administration.

Fig 6a The horizontal red line in Fig 6a shows the number of hypocretin cells in DTA mice maintained throughout on doxycycline, with 14 days of daily saline injection followed by sacrifice. The green bar shows mice with doxycycline withdrawal for 1.5 days followed by restoring doxycycline administration and saline injections for 14 days. A 30% reduction of hypocretin cells relative to the control level is seen. But when daily 100 mg/kg morphine injections were given for 14 days, the number of detected hypocretin cells was restored to baseline level (Blue bar). This difference was significant (p=0.003, t=6.31, df=4). Photomicrographs on right show examples of hypocretin labelling in saline (top) and morphine (bottom) treated animals. Cal 200µ.

Fig 6b Morphine administration greatly decreases cataplexy: Daily administration of morphine greatly reduces cataplexy in mice given chocolate after 1 and 2 weeks of administration relative to control saline injection. Treatment effect, F (1,6)=148.4, p=0.0001 †. Changes with saline administration are not significant. Post-hoc comparisons with Bonferroni correction revealed significant difference at P<0.01 between the saline controls and the morphine treated on both 1 week and 2 weeks of treatment. Saline treated narcoleptic mice has 9.2±1.2 cataplexies/hour and morphine treated narcoleptic mice had 2.8±0.6 cataplexies/hour with chocolate availability.

Fig 6c Human narcoleptic with cataplexy given over a long period morphine has higher number of hypocretin cells than control; case comparison. We identified a patient (NBB01064) diagnosed with narcolepsy with sleep paralysis and cataplexy 14 years prior to death, who had been treated with morphine 10 mg, 2-3x/day for 10 years for relief of her pain resulting from discopathy (blue). Eight years later the patient was reclassified as having idiopathic hypersomnia. The yellow bars in the figure show hypocretin cell counts in a diagnosed narcoleptic with cataplexy without longterm morphine treatment (yellow, NBB08023). The hypocretin cell counts of three other patients without narcolepsy or other identified neurological disorders are shown in green. All patients' brains shown in this figure were willed to the Netherlands Brain Bank and preserved and analyzed by the same techniques (Supplemental Table 2). Fx - fornix, Cal. 200 µm.

X

TABLE 1	Characteristics of Addicts, Narcoleptic and Control Subjects								
Subject	**Sex**	**Age**	**Cause of death**	**medications**	**PMI**	**pH**	**Time in**	**EST Hcrt #**	
Addicts (A)							fixative (years)		
A-1	M	38	Heroin intoxication	oxycodone abuse, smoked 1 & 1/2 pack per day	20.0	6.84	3.5	121173	
A-2	F	26	Asthma complicating Narc intoxication	heroin, cocaine, methadone (110g) for 8 months, non smoker	4.0	6.63	3.5	130719	
A-3	M	72	Cancer	former heroin addict, morphine alcohol "comfort" 4 days before death	19.0		3.6	111466	
A-4	M	28	Methadone intoxication and cocaine use	heroin, cocaine, methadone	13.0	5.52	3.0	123810	
A-5	F	19	Methadone intoxication	high dose drug abuse	24.0	5.45	3.0	141717	avg 125777
Control subjects for addicts (C)									
C-1	F	42	Breast cancer		15.0	N/A	1.0	84546	
C-2	M	32	MI, aneurism		N/A	N/A	8.0	89652	
C-3	M	61	Pneumonia, testicular tumor		N/A	N/A	8.0	65833	
C-4	F	44	Breast cancer		N/A	N/A	8.0	86354	
C-5	F	27	Mild hypoxia, arterial calcification, congenital heart defect, coronary artery atherosclerosis		N/A	N/A	1.5	78656	
C-6	F	33	SLE, sepsis		N/A	N/A	1.5	76921	
C-7	M	29	Leukemia, bacterial sepsis, hypoxia		N/A	N/A	1.5	89732	avg 81671
Control subjects for narcolepsy									
NBB-00-022	F	83	Acute myocardiac infarction		7.5	6.52	0.1	106366	
NBB-00-072	M	78	Kidney failure		18.0	5.84	0.1	88404	
NBB-00-142	F	82	Myocardial infarction		5.3	6.60	0.1	70976	avg 88582
Narcolepsy with cataplexy									
NBB-08023	F	66	Heart failure	Amphetamine 5.45mg 1 time/day),					
				Modafinil 100mg 3 times/day	7.0	6.57	0.1	3276	3.6% control
				Gamma-hydroxybutyric acid 30cc 2 times/day,					
				Sodium oxybaste 500mg/ml 2 times 6ml/day					
Narcolepsy with cataplexy - morphine treated - hypersomnia									
NBB-01064	F	85	Chronic pain syndrome with palliative sedation	Morphine 10mg 2 times/day (9 years)	3.4	6.77	0.1	16834	19% control
				Modafinil 200mg 1-2 time/day					

MORPHINE PELLET					
GROUP	DOSE	DURATION	N	GENDER	AGE (days)
Group I a	25mg/pellet	3days	2	M	114
Group I b	control pellet	3 days	2	M	114
Group II a	25mg/pellet	7 days	2	M	118
Group II b	control pellet	7 days	2	M	118
Group III a	25mg/pellet	14days	3	M	125
Group III b	control pellet	14 days	3	M	125
MORPHINE SOLUTION -14 days dose rsponse					
Group 1 a	1mg/kg	14 days	3	M	80
Group 1 b	saline	14 days	3	M	80
Group II a	5mg/kg	14 days	3	M	80
Group II b	saline	14 days	3	M	80
Group III a	10mg/kg	14 days	3	M	80
Group III b	saline	14 days	3	M	80
Group IV a	25 mg/kg	14 days	3	M	80
Group IV b	saline	14 days	3	M	80
Group V a	50mg/kg	14 days	3	M	80
Group V b	saline	14 days	3	M	80
Group VI a	75mg/kg	14 days	3	M	80
Group VI b	saline	14 days	3	M	80
Group VII a	100mg/kg	14 days	3	M	80
Group VII b	saline	14 days	3	M	80
MORPHINE SOLUTION -60 days dose rsponse					
Group I a	50mg/kg	60 days	5	M	142
Group I b	saline	60 days	5	M	142
Group I c	10mg/kg	60 days	4	M	142
Group I d	25mg/kg	60 days	4	m	142
MORPHINE ESCALATING DOSE					
Group 1a	100 mg/kg	7 DAYS	3	M	100
Group 1 b	100mg/kg escalating dose	7 days	3	M	100
Group 1 c	saline	7 days	3	M	100
Group II a	100mg/kg	14 days	3	M	107
Group II b	100 mg/kg escalating dose	14 days	3	M	107
Morphine for Western blot					
GROUP	DOSE	DURATION	N	GENDER	AGE (days)
Group Ia	100mg/kg escalating dose	14 days	4	M	164
Group Ib	saline	14 days	4	M	164
Group IIa	100mg/kg escalating dose	14 days and 2 weeks WD	4	M	178
Group IIb	saline	14 days and 2 weeks WD	4	M	178
Morphine for PCR					
Group Ia	100mg/kg escalating dose	14 days	8	M	164
Group Ib	saline	14 days	8	M	164
Morphine for Neurogenesis					
Group Ia	100mg/kg	14 days	3	M	164
Group Ib	saline	14 days	3	M	164

Morphine short term and withdrawal study					
GROUP	DOSE	DURATION	N	GENDER	AGE (days)
Group I a	50mg/kg	14 days	6	M	105
Group I b	saline	14 days	6	M	105
Group II a	50mg/kg	14 days & 2 weeks WD	5	M	119
Group II b	saline	14 days & 2 weeks WD	6	M	119
Group III a	50mg/kg	14 days & 4 weeks WD	6	M	133
Group III b	saline	14 days & 4 weeks WD	6	M	133
Group IV a	50mg/kg	14 days & 8 weeks WD	6	M	161
Group IV b	saline	14 days & 8 weeks WD	6	M	161
Group V a	50mg/kg	14 days & 16 weeks WD	6	M	217
Group V b	saline	14 days & 16 weeks WD	6	M	217
Group VI a	50mg/kg	14 days & 26 weeks WD	6	M	287
Group VI b	saline	14 days & 26 weeks WD	6	M	287
Morphine long term and withdrawal study					
Group I a	50mg/kg	60 days	5	M	142
Group I b	saline	60 days	5	M	142
Group II a	50mg/kg	60 days & 2 weeks WD	5	M	156
Group II b	saline	60 days & 2 weeks WD	5	M	156
Group III a	50mg/kg	60 days & 4 weeks WD	5	M	170
Group III b	saline	60 days & 4 weeks WD	5	M	170
Group IV a	50mg/kg	60 days & 8 weeks WD	5	M	198
Group IV b	saline	60 days & 8 weeks WD	5	M	198
Group V a	50mg/kg	60 days & 16 weeks WD	5	M	254
Group V b	saline	60 days & 16 weeks WD	5	M	254
Group VI a	50mg/kg	60 days & 26 weeks WD	5	M	324
Group VI b	saline	60 days & 26 weeks WD	5	M	324
DTA mice					
Group Ia	100mg/kg	14 days	4	M	83
Group Ib	saline	14 days	4	M	83

Hypocretin knockout (Hcrt-/-)					
Group Ia	100mg/kg	14 days	3	M	168
Group Ib	saline	14 days	3	M	168

Table 2. Characteristics of animals used for morphine study

We thank both reviewers for their careful reading of the manuscript and constructive criticisms. Our point by point responses are below

Reviewer: 1

In this paper, the authors examine the hypothesis that administration of opiates to humans or mice with narcolepsy with cataplexy (NC) due to loss of hypocretin neurons, can increase the number of hypocretin neurons, and prevent cataplexy attacks. They begin with their chance observation that an opiate addict had more hypocretin neurons than any other human brain, and then examine brains of other opiate addicts, with similar findings. They then replicate their findings in mice, by showing that there is an increase in the number and reduction in the size of hypocretin neurons in the hypothalamus in mice after treatment with morphine, with the maximum increase after 14 daily injections, and less with continuous opiates, or longer exposure. There is also a peak around 50 mg/kg dose, with lesser increases at higher or lower doses. The effect lasts around 4 weeks after opiates are discontinued, and is not due to neurogenesis. The same thing is found with other markers for hypocretin neurons (NARP, dynorphin). Systemic morphine was then found to increase firing of hypocretin neurons in vivo in rats. At this point, the trail becomes a bit fuzzier. The authors used a strain of mice that express the diphtheria toxin A under the hypocretin promoter, with a tetO configuration, so that the DTA is expressed only when doxycycline administration is interrupted. The authors use mice with 1.5 days off doxy, which have a 30% reduction in the number of immunohistochemical hypocretin cells. This was reversed by 14 d of morphine administration, and cataplexy was also reduced in these mice. The authors then go on (without even a paragraph break!) to describe a single case of a patient with NC who was "chronically treated with morphine" for spine arthritis for 9 years, and who apparently later (it is not clear when) [re-diagnosed after 6 years of morphine treatment, table 1] stopped having attacks of cataplexy. This patient at autopsy also had more hypocretin cells (16% of controls) than a typical NC patient (they compare this to a single NC patient who did not receive morphine, and who had 3% of hypocretin cells left at autopsy, not the 10% that has been shown to be more typical, and give no information on the range of hypocretin cells in patients with NC). This is reported in our prior study [1]. This then leads into a discussion about two previous similar case reports, and a single study of 27 NC patients, who had no change in their multiple sleep latency test. From this the authors try to make a case for a larger prospective study of opiates to treat NC.

The concept that different populations of neurons that we count due to their immunohistochemical staining may, in fact, have downregulated (or upregulated) protein expression, into the range where the immunostaining is effective or below it, is not a new one. In fact this same group (and Valko et al. Ann Neurol 2013; 74:794, in back to back papers) showed an increase in histamine neurons in narcoleptic brains. This has been a recurrent trope in immunohistochemical work, where showing that neurons disappear does not mean that they are dead, because they may have just stopped making enough of the target protein to visualize. Thus, it is not that surprising that such a change exists, and the novelty in this work is in showing the relationship with opiates. In that regard, the primary observation of the increase in hypocretin immunoreactive neurons with opiate treatment is fresh and interesting, and worthy of publication. On the other hand, the work trying to relate opiate treatment to improvement in symptoms in NC is not nearly as clean, and borders on dangerous (from the point of view of taking on an adversarial exhortative role, rather than reviewing the evidence, which is paltry and contradictory). Given that what the authors propose could lead to human trials, and the demonstrated danger of long term treatment of non-terminal patients with opiates (currently causing over 100 deaths per day in the US alone due to overdoses in addicted people), I think this part of it needs to be toned down, way down.

We are acutely aware of the risk of opiate use. A nephew of mine was one of the recent opiate overdose victims. The current work represents an important advance towards understanding opiate

addiction. I have altered the language and elaborate on the relation of the current work to both opiate addiction and narcolepsy below.

There are a number of points that would need to be addressed in revision:

1. In the introduction, the authors paint a surprisingly naïve portrait of themselves and the field. They clearly know that the demonstration of loss of hypocretin cells in NC was shown by Peyron and colleagues using in situ hybridization, not immune, so the statement in the abstract that NC is caused by loss of immunohistochemically identified hypocretin neurons (implying that this is the only evidence) is incorrect. They also ignore the Peyron paper in the Introduction. Second, given their paper on histamine neuron increases in NC brains, it is not "startling" that they would see something similar in the brain of the NC patients treated with morphine.
We removed the word “startled,” although that was our reaction when we saw the first case 5 years ago – long before our work with histamine cells. We now cite our original abstract, submitted to the Neuroscience meeting in 2012 [2]. The histamine work was published first because it is (according to our data) a phenomenon unique to human narcoleptics, therefore we could not pursue the nature of cause and effect in animal studies. We still do not know the cause of the greatly increased number of histamine cells in human narcoleptics [3]. In contrast, we were able to model the opiate induced change in the number of detected hypocretin neurons cells in animals over the 5+ years since our original finding and the results of this work are in the current paper.

The key issue in the paper is understanding the increased number of hypocretin cells detected immunohistochemically. This is why it is appropriate to mention this in the introduction and abstract, This is not to slight Peyron et al. We always reference Peyron et al.[4] when we reference Thannickal et al.[1] in our papers and in all our talks, unlike some others. We now present Peyron et al.’s in situ results in greater detail in the discussion and also discuss the *in situ* issue below, in this letter.

The authors then go on to give their results in the second paragraph of the introduction, drawing conclusions from them before presenting any of the actual data. Foul!!! This is the kind of hype that gives science a bad name.
We have removed this. But our goal was to emphasize and put the work into context in a way that could not fit in the abstract because of the word limit. We did not make any claims beyond what was in the abstract.

2. The data on NARP are given twice, once in the second paragraph of the Results (prematurely as there is no description of the situation) and then later in a dedicated section.
The first mention referred to the human Narp data and the second mention referred to the more extensive mouse Narp data. I have clarified the issue at the end of the second paragraph of the Results.

3. Parts of the Results section belong in the figure legends, not the text (e.g., the two complete sentences about fig. 5, at the top of p. 7 in the pdf). I have moved the Fig 5 text in the results to the appropriate place in the figure legend. Thanks for noticing this error. In the next paragraph, the authors did not use the new mice in the figure, they used them in experiments. Corrected. The 30% reduction in hypocretin cells is very odd to cause such severe cataplexy, which typically
is not seen in either mice or people who have more than 10% remaining neurons. How do the authors account for this? Does the DTA perhaps damage many of the remaining cells in a way that allows them to synthesize hypocretin, but not release it properly? The statement "The extensive

loss of most of the hypocretin cell bodies in the DTA mice more closely resembles the human narcolepsy anatomy and symptoms than the hypocretin knockout mice (7, 10))." is clearly disingenuous, as the mice had cataplexy and were used in this study at 30% hypocretin cell loss, which would cause no symptoms at all in other strains of mice or humans.
Sorry for this confusion. Two different groups of DTA mice were used in this study. One was the 30% depleted mice in figure 6a. The reason we used this level of depletion in our initial study was that our recently published colchicine studies revealed that more than 30% of the hypocretin cell population was not detectable with conventional immunohistochemistry [5]. We reasoned that if morphine increased the detection of hypocretin cells by increasing their production of the peptide, we might be able to reverse a 30% hypocretin cell loss. This turned out to be correct, as the figure illustrates. The second DTA group was depleted more completely to the 95% level that produces cataplexy (6b). This second study on DTA mice could only be done after Yamanaka's group studied the parameters of cell loss and the EEG and cataplexy consequences of this loss [6]. The parameters of depletion and related procedures used were described in detail in the Supplementary Methods section of our original submission and we have now added additional text to the Results section of the paper to prevent future confusion.

An important dataset that is missing in this paper is: what happened to the state instability (sleepy during the wake period, fragmented sleep during the sleep period) both in the DTA mice and in ones that were treated with opiates? How about orexin knockout mice, when treated with opiates. The fact that they did not have enough cataplexy is no reason for not measuring their wake-sleep parameters. Or was that done, and not reported here, because it did not change (or got worse) with the opiates? This is critical information if the authors are then going to endorse a human trial of opiates for NC. What if it makes the primary symptom (sleepiness during the day) worse, given that opiates tend to make people sleepy in the first place?
The state changes in the hypocretin 95% depleted DTA mice (without morphine) include cataplexy and disrupted daytime waking and nighttime sleep [6]. Our current focus is on determining optimal dose frequency and circadian changes in potency of morphine administered to the DTA mice on cataplexy and hypocretin cell count. Although we have extensive experience in recording EEG in mice, including hypocretin KO mice [7], we have not yet combined this with hypocretin depletion and morphine administration in the DTA mice. But we see no evidence of sleepiness after morphine administration. On the contrary, there is EEG activation and a marked increase in activity (figure 5). Mice were active for at least 6 hours after morphine injection at all doses tested, even when this was administered in the light normal sleep) period. They never slept after morphine administration. We now mention this in the Results.

The human narcoleptic data in Lancet [8] and Pharmacotherapy [9] reported that patients taking opiates for pain had, paradoxically, greatly increased alertness and reduced or eliminated cataplexy. This contrasts with the effect of opiates in neurologically normal humans. But this is consistent with our current results, indicating activation of hypocretin neurons by morphine, and with the now well established relation between increased hypocretin cell activity and increased arousal [1, 2, 4, 7, 10].

4. The case of the human patient who was treated with morphine for 9 years for benign pain is way over interpreted. First, most physicians would consider it to be incompetent care to provide morphine for 9 years for benign back pain. In the US, a physician would probably lose his/her license for a case like this. This reflects on the competency with which the original diagnosis of NC was made, and how strong the evidence was for cataplexy (given that the case was only examined for this AFTER the patient had died). 16% survival of hypocretin neurons would be quite consistent with hypersomnia, but unlikely to cause cataplexy. Comparing this with 3% survival in the one control case is also deliberately misleading, when the authors know (because they state it later) that usually there is

about 10% survival. 10% is not that different from 16% (given the large variance in remaining hypocretin neurons in NC patients), so claiming that this person had increased numbers of hypocretin cells, and then improvement in cataplexy on that basis is specious. Such a claim would not be permitted any reputable clinical journal.

None of the current authors were involved in the pain treatment of this patient with opiates. Drs. Lammers and Fronczek, who made the diagnosis of narcolepsy with cataplexy more than 10 years before the patient's death, are two of the world's leading narcolepsy researchers, with extensive clinical experience and many important publications on human narcolepsy. There is no reason to doubt the accuracy of their diagnoses. As the reviewer points out the longterm treatment with opiates for pain is ill advised, but it is very common. The encouragement of physicians to administer longterm opiate treatment for pain by certain drug companies is one of the major causes of the epidemic of opiate deaths [see http://www.nytimes.com/2016/05/07/opinion/the-opioid-epidemic-we-failed-to-foresee.html?action=click&pgtype=Homepage&clickSource=story-heading&module=opinion-c-col-left-region&region=opinion-c-col-left-region&WT.nav=opinion-c-col-left-region&_r=0].

The morphine treated patient (NBB-01064) , reclassified as having idiopathic hypersomnia- had 9% of the control number of hypocretin cells in our paraffin sectioned controls, vs. 3% of the control number in the in the paraffin sectioned non-morphine treated narcoleptic with cataplexy patient (NBB-08023). As indicated in the Supplementary Methods, the counting system and histological preparation differed between the frozen sections that we previously used and paraffin sections on which this patient's brain had been preserved, so they cannot be directly compared.

The case of this patient and a control patient with narcolepsy sectioned stained and counted (blindly) at the same brain bank and laboratory is unique. It is an unplanned "experiment" conducted on a patient who had narcolepsy and also willed her brain to research. It is unlikely that many more such cases will be found since it represents an intersection of several rare events. The incidence of narcolepsy is 1 in 2,000 (in the US). The total number of identified human narcoleptic brains in the world is about 40. The likelihood that identically handled, sectioned, stained and counted narcoleptic brains, some from patients treated with opiates over a long period, and suitable narcoleptic and normal controls will be identified elsewhere is vanishingly small. The correlation with symptom diminution and the relatively high number of detectable hypocretin neurons in the morphine treated patient is striking and important.

The progression of narcolepsy is typically from initial sleepiness to sleepiness with cataplexy. Once cataplexy is established it is typically thought to be lifelong. To our knowledge, no one has ever "recovered" from narcolepsy. This patient did, to the extent that the diagnosis was revised (long before the current analysis was undertaken) to idiopathic hypersomnia. So three unusual events occurred in this patient, reversal of the cataplexy diagnosis, longterm opiate administration and higher hypocretin cell counts than the only other patient whose brain was available for comparison. We also see that opiate administration greatly reduces cataplexy in mice depleted of 95% of their hypocretin cells. For these reasons this case and the narcoleptic and normal "controls" that we analyzed with it are uniquely instructive. We intentionally did not mention this finding in the Abstract, Introduction or Discussion (and put it within a longer paragraph in the results) because we had the same concerns as the reviewer, and did not want to "hype" our conclusions as definitive. However, leaving these unique human neuropathology findings out of the paper would be a disservice to the field. I have now elaborated and cautioned on this issue in the Results. In isolation the human case report studies (using two narcoleptics and three controls, must be viewed in the context of our very extensive human heroin addict data and the extensive and systematic mouse data, all of which show well documented, similar changes. Also see our mention of the value of showing surviving hypocretin neurons below.

5. In the Discussion, the authors claim that immune is the most sensitive method for identifying and quantifying neurons. It is certainly the most convenient method, and the most widely used. But I would argue that in situ hybridization is generally more sensitive, and since this method has already been used, successfully in the Peyron study in NC in humans, ignoring it is simply misstating the literature. I also disagree with the statement that "the additionally recruited neurons are not uniformly distributed across the population of hypocretin cells, suggesting that they may receive a different pattern of inputs, may project to a different pattern of sites and may differ in other ways from the populations observed prior to opiate administration." First the additional neurons are not that unevenly distributed (fig. 2d), and second, such distribution says absolutely nothing about inputs or outputs of these neurons.

The in situ technique requires an arbitrary cut off and is good at comparing identically treated experimentals and controls. But depending of the threshold set, the counts will vary. The Peyron et al paper [4] reported 15,000-20,000 hypocretin cells in human controls (less than the number they reported in normal dogs [11]) and zero hypocretin cell numbers in human narcoleptics. Their 15-20,000 control number is much less that our initial 70,000 count with immunohistochemistry which has been replicated repeatedly. The website of the Stanford Sleep Center [https://stanfordhealthcare.org/medical-conditions/sleep/narcolepsy/symptoms.html], which produced the Peyron et al paper, now lists our 70,000 number rather than their 15-20,000 as the number of hypocretin neurons.

Other studies have subsequently replicated our finding that all human narcoleptics have a considerable number of surviving hypocretin cells, averaging 10% (~7,500) of the control level. Although our 2000 paper has been cited 1,640 times, I am not aware of any publication indicating that human narcoleptics do not have any hypocretin cells apart from Peyron et al., 2000). The complete lack of immunoreactive cell bodies in hypocretin KO mice verifies the specificity of our immunohistochemical technique, indicating that if the cells were absent in human narcoleptics, investigators would have verified this with immunohistochemistry or other techniques [3, 5, 7]. The presence of surviving hypocretin cells in human narcoleptics is as important in understanding the etiology of, and in developing new treatments for, this disease as was the original discovery of hypocretin cell loss. Our current study provides yet another piece of evidence of surviving hypocretin cells in human narcolepsy (Fig 6c), using preservation, sectioning and immunohistochemical techniques differing from most prior studies. In response to the reviewer's comment we now add a brief mention of these differences between the cell counts of Peyron et al.[4] and Thannickal et al. [1] in the Discussion.

I agree that the differences in the distribution of hypocretin cells in opiate treated human and mice proves nothing about inputs and outputs. But it does not seem much of a stretch to think that cells that do not produce detectable levels of hypocretin under baseline conditions may differ in their inputs and outputs from those that do not. This is all we said. Even if they had been uniformly intermixed, which was not the case, understanding whether the cells that do not stain under baseline conditions, but do after morphine administration, might differ in projections or in other aspects is potentially quite important. We are investigating this aspect with some encouraging initial results.

6. The discussion of the human literature is irresponsible, and should not be permitted, especially given the obvious ramifications. Two case reports, in human work are just that. There are about 100,000 patients with NC in the US, and most of them have probably been treated with opiates at one time or another. It would seem that if improvement in their symptoms was common with opiates, this would have much more widespread reports (or were these two patients who claimed they were better just drug-seeking?). The sole clinical trial, while underpowered, failed. This is not a ringing endorsement of the concept that NC could be improved with opiates. Perhaps a tentative suggestion

in this direction could be made, but the text here reminds me of a greedy defense lawyer trying to make the case for his client, even knowing how dangerous a murderer that client is. Opiates kill. They kill more Americans than auto accidents. Uncritical calls for testing opiates in benign, chronic conditions are simply irresponsible.

Implying that we are like greedy lawyers and irresponsible is a bit much. Our goal is to understand heroin addiction, which has caused over 300,000 deaths in the US since 2000 and to improve treatment for human narcolepsy which affects approximately 150,000 Americans.

Regarding the reviewer's first point, the two studies mentioned in the Discussion demonstrate reduction in cataplexy and increased alertness in human narcoleptics treated with opiates. The "underpowered," single dose level, 1 week study that ended the prescription of opiates for narcolepsy reported that all the patients' symptoms improved. They did not quantify cataplexy. But because the multiple sleep latency test results were not significant the authors emphasized that the treatment was not effective [12].

The possibility that the patients did not really have symptom reversal and "were just drug seeking" cannot explain the dramatic reduction of symptoms we report in the narcoleptic mice.

Nowhere in the paper do we suggest that that narcoleptics should try or physicians prescribe opiates to their narcoleptic patients. We do point out that further studies need to be done and that the earlier human trials were not adequate.

We had the same thought as the reviewer about whether there was accidental patient self-discovery of the benefits of opiates, beyond those in the two initial papers. So we Googled "opiates and narcolepsy" and found that many narcoleptic patients are taking opiates and finding it effective in decreasing cataplexy and increasing alertness. They are discussing this drug use on the web, suggesting that even higher numbers may be taking opiates more discretely [see attached], even though it is not an accepted treatment for narcolepsy and most likely was not prescribed for narcolepsy. Because of the negative paper by Fry et al.[12] and the risk of prescribing opiates, it is unlikely that many physicians would give a schedule II drug for such "off-label" use. We hope to eventually conduct a clinical trial, but only after further animal studies and have now again emphasized in the discussion that we do not advocate opiate self-treatment.

Most narcoleptics routinely take highly addictive drugs daily. A very common treatment for the sleepiness of narcolepsy is methamphetamine. The Centers for Disease Control reported that there were 4,298 psychostimulant deaths in the US in 2014-up 229% from 2004, at the height of the methamphetamine epidemic.

Another highly addictive drug typically prescribed for narcolepsy in gamma hydroxybutyrate (GHB, sodium oxybate, Xyrem). Although the US death rate has not been characterized, toxicologists at Imperial College London analyzed data from 2011 to 2015 and found that the number of GHB deaths in London more than doubled in just 12 months to 29 [13]. This would translate into an annual death rate in the US of 1,087.

Many narcoleptics take both stimulants and GHB. Although these drugs are helpful, no combination of these drugs adequately treats the symptoms. Both treated and untreated narcoleptics have a very high level of disability and impairment.

So although opiates, because of their availability and toxicity, are currently in a class by themselves, with 60,000 annual deaths and over 300,000 deaths since 2000, up from 18,000 in 2000, other less

readily available drugs with substantial overdose death rates are in daily use by narcoleptics. Narcoleptics have not been reported to abuse these drugs and I am not aware of any deaths from drug overdose in narcoleptics. Certainly such deaths are not very common. It was only by convincing the FDA of the low to non-existent rates of drug abuse by narcoleptics that approval for GHB use for narcolepsy was given. It continues to be highly effective and safe for narcoleptics. We have speculated in the Discussion that the lack of abuse in narcoleptics is precisely because of the loss of hypocretin cells that underlies narcolepsy. Whereas our data indicates that the greatly increased hypocretin cell count described in our paper may be a substrate of addiction, the hypocretin cell loss in narcolepsy may protect against abuse. Hypocretin deficient animals have reported to be resistant to addiction. Harris et al. showed that hypocretin was required for learning a morphine conditioned place preference task[14, 15]. Constitutive hypocretin KO mice developed attenuated morphine dependence, indicated by a less severe antagonist-precipitated withdrawal syndrome [16]. Orexin receptors activate the mesolimbic dopamine pathway and related behaviors induced by morphine [17]. Although caution is required in opiate use, even with the catastrophic results from abuse there is still a place for it in the treatment of pain. Likewise, in the special situation of narcolepsy, characterized by loss of hypocretin cells, opiates at the correct dose and administration schedule may be a more effective treatment, perhaps substituting for the current drugs. Beyond this, opiate analogs, such as methadone may be effective without the same risk as morphine. We are currently testing this in mice. Further down the road our findings may lead to the development of non-opiate drugs that more specifically target hypocretin cells. We are currently using transgenic techniques to explore this possibility.

So we see these results as the start of a journey to a better treatment of opiate addiction and narcolepsy, not as the endpoint. We have made this clearer in the discussion.

We include the following patient comments about opiates and narcolepsy in response to Reviewer One's question about why, if opiates are effective against narcoleptic symptomatology, this has not been accidentally discovered by narcoleptics. "There are about 100,000 patients with NC in the US, and most of them have probably been treated with opiates at one time or another. It would seem that if improvement in their symptoms was common with opiates, this would have much more widespread reports." "Testimonials" on the web are consistent with the two published reports about the reversal of cataplexy and sleepiness with opiates cited in the Discussion. One can assume that if so many individuals have publicized their possibly illegal "off-label" use on the web many more use it discretely. Here are a few of the patient comments found on the Web. .

https://www.drugs.com/answers/has-anyone-heard-of-opiates-for-treatment-of-448301.html

Home › Q & A › Questions › Has anyone heard of opiates...

Has anyone heard of opiates for treatment of Narcolepsy? CNS stimulants just don't work at all?

Asked

5 Sep 2011 by **booanjel**

Active

7 Oct 2011

Topics

CNS stimulants, Ritalin, Adderall, Provigil, dextroamphetamines, even mixes of them just don't work. I broke my leg, was prescribed hydrocodone 10- 325s. I had never felt so awake in my life since Narcolepsy! I am 47 and was diagnosed when I was 23. It was an amazing feeling! I could stay awake for a movie, or car ride! I could just " stay awake"! No fast heartbeat, or crashing! No anxious feelings. They were truly a blessing.

Answer this Question

Report Favorite

Responses (6)

LA

LaurieShay 5 Sep 2011

Hey booanjel,

I have never heard of a doctor prescribing opiates for narcolepsy but I have heard of opiates affecting people the way they do you, keeping them awake rather than the more common making them drowsy. Only problem is going to be getting a doctor to prescribe an opiate longterm without an ongoing pain need. Have to discuss this one with your doc.

Best wishes,

Laurie

JP

jpelektrk 17 Sep 2011

I am 29 and have been living with narcolepsy all of my adult life. I am currently prescribed 400mg of Provigil, 300mg of Welbutrin, and 10mg of Ambien. The Ambien is obviously to help me sleep through the night. Even with all of these meds, I get very little relief from EDS and cataplexy. A couple of years ago I began using hydrocodone to help stay awake. I only use it on days that are very important to stay awake for, like work meetings, family functions, or days that my usual sleep pattern just isn't an option. I usually take it once or twice a week. It is very effective for staying awake, and makes it much easier to sleep through the night. It doesn't seem to help the cataplexy. I am not prescribed hydrocodone. On the days I take it I use about 15-20mg to stay awake through the day. The wakefulness last long after any euphoria, or high would be causing.

I can take it in the morning and still be awake in the evening, so I doubt it's a "euphoria" that is causing the wakefulness. I doubt any doctor would prescribe hydrocodone for narcolepsy, which is very unfortunate seeing as how they feel drugs like xyrem(aka GHB/ the date rape drug) are a reasonable option. The drugs commonly prescribed for narcolepsy are less addictive than hydrocodone, but have just as damaging, if not worse side effects. The fear of addiction doesn't bother

me since I have to accept the fact that I will always have narcolepsy and always be on multiple drugs for it. I hope someday doctors will take a serious look at how hydrocodone effects narcolepsy. Until then I will continue to struggle to stay awake through life, with the exception of the days I take hydrocodone.

February 2, 2014 | ehealthme.com
"Hey, there's a not so common symptom associated with ...High doses of **Ritalin** helped me quite a bit, and 15mg of **vicodin** a day completely relieved my symptoms for both **narcolepsy** and cataplexy."

psychonauticat

- Gender:Female
- Interests:Pharmacology, chemistry, arts and crafts, cats, playing various instruments.

Posted March 1, 2015
Recently my cataplexy has been very bad, Ferret mentioned in another thread that I may be experiencing rebound cataplexy since I had to discontinue using cannabis I had been using to self medicate for it which I suspect is true. Now that my diagnosis was confirmed I had to taper off my self medicating under directions by my doctor before I could try Xyrem.
This part has nothing to do with N+C but I also have poly cystic ovaries and was in the ER the other day for a ruptured cyst. They sent me home with a prescription for oxycodone 10mg 3x day for the pain until it subsides. Not percocet, instant release oxycodone since I can't take acetaminophen for different reasons.
The part that does have to do with N+C is that since I had been taking the oxycodone for the pain my cataplexy is mostly gone and my EDS is reduced. It almost feels like the oxycodone itself is a mild stimulant. Can anyone more experienced in this realm shed some light? Obviously this isn't an approved treatment nor am I suggesting it to anyone as a method to control their cataplexy, I'm just wondering why it suddenly stopped the severity of my episodes.
Edit: By stopped I mean I've gone from having multiple daily episodes to only a single knee buckling and one full body collapse while laying down after my cat jumped on me in the past 3 days.

allright
Member
I think the topic has been confused but at least Oxycontin has been exposed for its potential and addictive properties.
WHEN IT IS ABUSED OR TAKEN FOR TOO LONG A PERIOD
Don’t short change PWN they are not idiots but are well educated about medication. . Xyrem, amphetamines, provigil, are also dangerous drugs. Oxycontin is not crack. Most people are prescribed Oxycontin don’t get hooked but if you don’t manage it – it will manage you.
Oxycontin is really only a problem if you make it one. We are talking about a drug that is given to all patients with serious pain. It’s not a closet drug. It’s nothing to fear unless you don’t understand its properties.
You take it and get off it as quick as you can. I am sorry if I have caused you anxiety or fear talking about this drug. I should have been more clear that it of itself is not dangerous but how it is taken, for how long and its strength of dosage.

Just a point of interest regarding your point of view on dangerous drugs
Your point of view of Oxycontin in contrast to Xyrem is strange
Xyrem is only prescribed in the USA and other countries deem it too dangerous to prescribe and is unproven in studies.
USA is quick to bring a new drug on the market and more reluctant to take one off then other countries.
This why you can only get Xyrem from a central pharmacy.
I am trying to explain to you that your fear of oxycontin is valid but unfounded to speak of when Xyrem is being spoken about so freely. Oxycontin like Xyrem is only dangerous when used incorrectly.
What is the most important information I should know about Xyrem?
Xyrem is a federally controlled substance.
This means that if you sell, distribute, or give your Xyrem to anyone else, or if you use your Xyrem for purposes other than what it was prescribed for, you may be punished under federal and state law by jail and fines.
It is very important to keep Xyrem out of the reach of children and pets. Get emergency medical help right away if a child drinks your Xyrem.

Xyrem can cause serious side effects including trouble breathing while asleep, confusion, abnormal thinking, depression, and loss of consciousness. Tell your doctor if you have any of these problems while taking Xyrem.
The active ingredient of Xyrem is gamma-hydroxybutyrate (GHB). GHB is a chemical that has been abused and misused. Abuse and misuse of Xyrem can cause serious medical problems, including seizures, loss of consciousness, coma, and death. Abuse of Xyrem can lead to dependence, craving for the medicine, and severe withdrawal symptoms.
Your dose may need to be adjusted have sleep apnea, snoring, breathing, or lung problems. You may have a higher chance of serious breathing problems with Xyrem.
are on a salt-restricted diet, have high blood pressure, heart failure, or kidney problems.
Xyrem contains a lot of sodium (salt) and may not be right for you.
are pregnant or plan to become pregnant. It is not known if Xyrem can harm your unborn baby.
are breastfeeding. It is not known if Xyrem can pass through your milk. Talk to your doctor about the best way to feed your baby if you take Xyrem.
Tell your doctor about all the medicines you take, including prescription and non-prescription medicines, vitamins, and supplements. Especially, tell your doctor if you take other medicines to help you sleep (sedatives). Sedatives should not be used with Xyrem.
The most common side effects with Xyrem are nausea, dizziness, and headache, vomiting, sleepiness and bed-wetting. An increase in side effects may happen with higher doses.
Just a point of interest regarding your point of view on dangerous drugs
Your point of view of Oxycontin in contrast to Xyrem is strange
Xyrem is only prescribed in the USA and other countries deem it too dangerous to prescribe and is unproven in studies.
USA is quick to bring a new drug on the market and more reluctant to take one off then other countries.

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So about ten years ago I was diagnosed with narcolepsy with cataplexy and have been trying everything under the sun to sleep at night and stay awake in the day to no avail. The best they could come up with was citalopram and Ritalin and ambien. Got rid of most of the noticeable cataplexy but left me feeling
foggy and unmotivated, like a zombie. About a year ago I had a surgery to re-break and set a broken leg. Not wanting to mix drugs I had stopped taking everything prior to the surgery. After the surgery, I was on opiate painkillers for about six months and didn't have a single episode of cataplexy, was alert and motivated during the day and tired and able to sleep well at night. As my leg healed I begin decreasing the dosage gradually and eventually stopped altogether. Within 24 hours I had several major cataplexy episodes, and the EDS and insomnia set back in. This went on for a week and a half because I didn't correlate the two. After a particularly painful physical therapy session I took a few vicodin for the pain for the next few days. The cataplexy went away again, as well as the EDS and insomnia. This time my brother noticed the correlation and we experimented, and sure enough when I would take it, no problems with any symptoms. When I would stop, within 24 hours the symptoms would all return. I told my doctor this, and if course he just thought it was a ploy to get more vicodin, but he at least humored me and ran sleep studies both on and off the painkillers. After that he was convinced and began writing me prescriptions for low doses of vicodin 7.5/325 2x daily, and it's eliminated the cataplexy. And relieved most of the EDS. Still left me with trouble sleeping, but not nearly as bad as before. Now, the catch is that he wants me to revisit a few alternatives that I already tried with the previous doctor. Enter xyrem. So I stopped the vicodin and started the xyrem at night and Ritalin in the day. 2.25g diluted in water. Twice a night. Problem is, it does very
little for me. I get maybe an hour or two of sleep a night. And a week in not only have a not seen any positive results, I have actually seen very negative results. My EDS is crazy, cataplexy is in full on fall-flat-on-my-face-when-I- tell-a-joke mode... I have been reading a lot of negative things about xyrem on here, and I remember the first time I took it back a few years ago not getting any results from it...
So, has anyone else taken opiates and experienced relief from symptoms? I realize it's not an approved treatment for it, but stuff happens and there has to be another narcoleptic out there who has been on painkillers at some point.

Reviewer: 2

This is an important paper reporting a major breakthrough in the field of narcolepsy. Obtained results have major implications that can help understanding the functional modulation of neurotransmitter expression.

In fact, this study demonstrated for the first time that administration of opioid agonists increases the number of cells expressing hypocretin in the hypothalamus. The effect is specific for hypocretin and is not present for MCH, produced by neurons in the same hypothalamic region. This opens the door to the possible use of opiate receptors agonists for narcolepsy therapy or other neurodegenerative diseases with decreased production of hypocretin.

Equally interesting, in my opinion, is the finding that the increase in hypocretin neurons is not due to neurogenesis, suggesting that in physiological conditions there is a neuronal population whose function can be modulated by neurotransmitters, neuromodulators or pathological conditions.

Such modulation could indeed be a phenomenon with a general value, and not be a peculiarity of hypocretin neurons. This could then disclose discordant data in the literature about the consistency of neuronal populations in pathological conditions, such as that of histaminergic neurons in narcolepsy.

I only have a concern about mechanisms. It seems reasonable that in narcoleptic patients there may be a population of neurons not expressing HCRT because of the disease, and that opioids may induced HCRT expression in these neurons. However, it is more difficult, at least to me, to imagine how treatment with morphine can increase the number of neurons that produce HCRT in animal models of narcolepsy. Transgenic mice (Atx3 or DTA) have toxic necrosis of neurons produced by toxins co-expressed with pre-pro- HCRT. If the neurons are dead, how can morphine increase the expression of HCRT? A 2-week suspension of doxycycline produces a very strong reduction in the number of HCRT neurons (5% of controls) that persists for several weeks after the re-introduction of doxycycline (Tabuchi 2014). It is difficult therefore to think having a population of neurons damaged by the toxin but still alive that can be reactivated by morphine. Authors should argue on this point.
Clearly, we are not creating new neurons. Although we think that some apparent cell “loss” in neurological disease may in fact be the result of downregulation of expression of the identifying antigen, it is unlikely that this is the sole or even majority of the cell loss seen immunohistochemically. The elevated expression of gliosis in the hypothalamus of narcoleptics that we reported is evidence for at least some degeneration[1]. We still don’t know why degeneration occurs in human narcolepsy, and why some cells always survive, but some cells may not be vulnerable for reasons not yet determined.

In the DTA mice the expression of diphtheria toxin is under the control of the hypocretin promotor. One should expect that the cells surviving after a limited period of DOX withdrawal may have survived because they have less expression of this promotor and therefore less hypocretin and less tetanus toxin expression when DOX is removed. As Tabuchi et al. have shown, after DOX is restored there is no further loss of hypocretin cells, suggesting that the toxin is metabolized or secreted from the surviving neurons. Alternatively, the hypocretin neurons that survive short periods of DOX removal may be more resistant to diphtheria toxin itself. In either case hypocretin cell loss is progressive, rather than immediate. This variability must be the case, because a short withdrawal of doxycycline does not kill all the cells and there is a fairly regular relation between the length of time off DOX and the percent of hypocretin cells lost as is documented in the Tabuchi et al.. To more directly address this reviewer’s interesting point, we would predict that morphine administration after very longterm removal of doxycycline which caused all hypocretin neurons to disappear would not produce more

detectable hypocretin neurons or at the very least would produce a much smaller increase than in wild type animals.

Seeing the hypocretin cells population as on a continuum of hypocretin promotor expression or toxin sensitivity is novel, since phenotypically characterized neurons are typically treated as an identical cohort. We find this concept exciting for its ability to not only explain the phenomena we are reporting here in mice and in human heroin addicts, but to also explain recovery of function after damage and perhaps other aspects of brain plasticity. Similarly in human narcoleptics we know that there are always surviving hypocretin neurons and presumably, from our recently published study with colchicine [5] a large number of neurons capable of producing hypocretin that is not seen under baseline conditions, but can be activated by opiates, as in the heroin addicts.

Specific remarks:

Introduction, page 3. The first brain examined is that of a “former” heroin addict. How long had the heroin been suspended? This information would be useful to understand how long the effect outlasts administration of heroin in humans.
According to his records, he stated that he stopped heroin use 10 years before death. But his record also indicates administration of a single “comfort dose” of morphine and alcohol 4 days before death. We have now added this to Table 1. Our studies in the mice (see fig. 2) suggest that this is unlikely to have affected his hypocretin cell number, since even with maximal doses, we saw changes in hypocretin cell number only after 2 weeks of daily dosing (not after one week). It is striking that the magnitude of the increase in human heroin addicts is greater (55%) than that in mice even at optimal doses (40%). This suggests either a small species difference or the difference between a single bout of usage in the mice and repeated usage titrated to produce optimal pleasure in humans over years. We plan to study the effect of repeated bouts of morphine administration in mice to simulate the human pattern in the future.

Results, page 4. Cause of death of two of control subjects for addicts (Table 1) is breast cancer; have these patients never taken opiates to control the pain?
Because of HIPPA restrictions we do not have detailed information on the treatment of these patients, except that they were neurologically normal, non-addicts. In our prior studies we have not seen a single patient with the high numbers of hypocretin cells that we see in all human opiate addicts despite cancer and other painful illnesses prior to death, although we, similarly, do not always have a full report on pre-mortem drug use.

Results, page 4. Hypocretin cells are 22% smaller in the addicts (Figure 1 b): this point is never really discussed by the Authors. What significance could be the decrease in cell volume, which is specific to Hcrt neurons, not affecting MCH neurons?
We believe it is related to the very high rate of discharge of these neurons elicited by morphine administration (Fig 5). This is the first documentation of this increased activity with morphine. The shrinkage is extremely robust, even more rapidly developing than the cell number increase. One may speculate that the greatly increased activity that we see in hypocretin neurons with morphine may have the opposite effect on cell size as peptides/proteins including hypocretin, Narp and dynorphin transported out of the neurons and down their axons faster than they can be synthesized and ionic pumps work to restore membrane polarization, thereby depleting the substrates for supporting these functions and shrinking the cells. We now briefly mention this possibility.

Results, page 4,5. 60 days administration produces an increase in the number of highlighted neurons HCRT smaller than 14 days administration, the most effective dose (25 mg / kg) is lower (50 mg / kg

for 14 days), and also the duration of effects after termination is shorter (Figure 2c, e). This might suggest that in the mice, unlike in humans (where a 50% increase has been seen in subjects addicted presumably for many years), the effect tends to run out over time, as if prolonged exposure to high doses exert a toxic effect on Hcrt cells. This is also suggested by the effects of pellet administration, which increases morphine levels with respect to phasic administration. In this case, there is an early decrease in cell volume (cellular suffering?) and the number of neurons expressing Hcrt does not increase. What is the opinion of the Authors on this point?
We cannot rule out the possibility that some hypocretin cells die. But we never saw a decrease below baseline level even with longterm administration, after longterm withdrawal. Therefore, we think it is likely that an adaptation occurred to the metabolic effects of morphine, rather than death in hypocretin cells. This results in more "hypocretin cells" being taken "off-line." If high doses were toxic, we might expect that the number of hypocretin neurons in human addicts, who have often been taking opiates for years, would be less than controls, rather than the large increase we see. The reviewer raises the interesting possibility that other cell populations may die or cease functioning with addiction. This would be a very interesting subject for future studies.

Results, page 6. Figure 4b shows no significant changes in BrdU labelled neurons after morphine (no neurogenesis). However, neurons are counted in the perifornical area. In this region, there is not any significant increase in the number of Hcrt cells (Figure 2d): the count BrdU labelled neurons must be done in LH, where the increase of the neurons is maximum, not in PFA, where it is not detectable.
Sorry, we now more clearly indicate that the bar graph in 6b covers the entire hypothalamic hypocretin field, region including medial, lateral and perifornical regions and shows a nonsignificantly smaller number of BrdU cells in the morphine treated mice. In examining this figure we realized there was a typo in the labelling of 4b and have corrected it. The BrdU labelled cells are not necessarily hypocretin neurons; rather they are newly formed cells of any type. We chose to show the histology from the perifornical region because it contained the highest number of BrdU cells in both control and experimental subjects, though still a very small number, order of magnitudes smaller that the increase in hypocretin cells detected after chronic morphine administration. The doublecortin staining (to identify immature neurons) in 4c shows a complete lack of staining and is independent confirmation that the hypocretin cell number increase was not due to neurogenesis.

Results, page 7. DTA mice receiving morphine also receive chocolate at 6PM, like during baseline?
Yes. Which is the number/hour of cataplexy episodes in saline injected mice? 8. 9.2 ±1.2 episodes/hour in the controls. In the morphine group 2.8 ±0.6/hour. We now add this information to the figure 6b legend.

Have these animals been studied for sleep pattern? It would be interesting to know the effect on wake fragmentation. In fact, in narcoleptic patient under morphine therapy, cataplexy disappeared, but hypersomnia remained (idiopathic hypersomnia without cataplexy).
Yamanaka's groups extensively studied the changes in Hcrt cell number with cell depletion in DTA mice [6] and their sleep pattern (but not the opiate effects). As we see in figure 5, morphine administration strongly increases waking, an effect lasting several hours. All mice had continuous waking after morphine injection at all doses tested for >3 hours. Also, in the two published studies mentioned in the Discussion, human narcoleptics uniformly reported increased waking and alertness after morphine, a paradoxical effect considering that morphine is soporific in normals. [We also saw this in the anecdotal reports on the Web (see above) in response to Reviewer 1's question about why patients have not discovered this effect themselves – they have.]

In general, hypersomnia is the first symptom to appear in narcolepsy, followed by cataplexy, with this symptom appearing months to several years later. We can speculate that boosting Hcrt neuronal

function with intermittent opiate administration in this patient incompletely reversed the process. We are not aware of any other human narcoleptic with cataplexy whose diagnosis was “revised downward” in the way that apparently happened in this patient. I have asked several of my colleagues if any of their narcoleptic patients had ever recovered from the disorder. The consistent answer is that “this never occurs,” although treatments reduce symptoms.

We are planning on doing further studies of EEG changes in the mice now that we have explored the various dose/duration effects. But please appreciate that the reported results (including 35 different experiments as indicated in our figures) are the work of several full time investigators working for over 5 years. We are constantly assessing and selecting the highest priority studies.

Results, page 8. Both brains of narcoleptic patients have a percentage of HCRT neurons close to that described in the literature (85% -95% reduction) (Thannickal 2000). It would be desirable to increase the number of subjects to confirm the data. If this is not possible, to specify that, although the data is suggestive, both values are normal for narcoleptic patients.
We now elaborate on this.

All of our work and most of the rest of the work on Hcrt cell numbers in human narcolepsy have been based on immunohistochemistry on 40 µ sections and computer assisted counting of cells. The brain tissue from the Netherlands brain Bank are thin 6µ thick paraffin sections using different antibodies and counting procedures developed by Dr. Swaab. To deal with these variables we used age and sex matched controls from their inventory, all prepared in the same way. The narcoleptic not given opiates had a 97% lower count of hypocretin neurons whereas the morphine treated subject had 81% of the matched controls preserved, sectioned, stained and counted in the same way. I am not aware of any other cases in which a correctly diagnosed narcoleptic with cataplexy patient stopped having cataplexy. So these two phenomena certainly fit in with our human heroin addicts having more hypocretin cells, the mice administered opiates having more hypocretin cells and the narcoleptic mice having markedly fewer attacks when given hypocretin. The key claims in the paper are not dependent on the 3 controls and two patients from the Netherlands Brain Bank. But they are a virtually 1 in a million event that should not be hidden. We did not mention this data in the Abstract, Introduction or Discussion because we do not consider it definitive. We point out that these data are not definitive. But I think we would be derelict in not reporting this part of the story. The two subjects and 3 controls are unique “one of a kind” finds, not likely to replicated soon, if ever, in contrast to our mouse and human opiate data. We now state clearly that we do not consider these results to be definitive.

Methods, page 22. It is not specified that female DTA mice are implanted with electrodes for detecting EEG and EMG. I assume electrodes are there, because it is said that cataplexy was scored based on consensus criteria (presence of nuchal atonia, theta activity, 40 seconds of continuous wakefulness preceding the episode), and it would not be possible to do so without EEG and EMG.
These mice were not implanted. But cataplexy can be scored from recorded close-up video with the criteria adopted from a consensus conference in which we participated[18] and used in previous studies [18-21]. The scoring of the attacks was done by the same individuals who worked with implanted wild type and KO mice in the past. Importantly, the use of video allowed the scoring to be done entirely blind to condition.

Reference List

1. Thannickal,T.C. *et al*. Reduced number of hypocretin neurons in human narcolepsy. *Neuron*. **27**, 469-474 (2000).

2. Thannickal,T.C. *et al*. Human narcolepsy is linked to reduced number, size and synaptic bouton density in hypocretin-2 labeled neurons. *Abstr Soc Neurosci* **26**, 2061 (2000).

3. John,J. *et al*. Greatly increased numbers of histamine cells in human narcolepsy with cataplexy. *Ann Neurol*. **74**, 786-793 (2013).

4. Peyron,C. *et al*. A mutation in a case of early onset narcolepsy and a generalized absence of hypocretin peptides in human narcoleptic brains. *Nat. Med*. . **6**, 991-997 (2000).

5. McGregor,R., Shan,L., Wu,M.F., & Siegel,J.M. Diurnal fluctuation in the number of hypocretin/orexin and histamine producing: Implication for understanding and treating neuronal loss. *PLoS ONE* **12**, e0178573 (2017).

6. Tabuchi,S. *et al*. Conditional Ablation of Orexin/Hypocretin Neurons: A new mouse model for the study of narcolepsy and orexin system function. *J Neurosci* **34**, 6495-6509 (2014).

7. McGregor,R., Wu,M.-F., Barber,G., Ramanathan,L., & Siegel,J.M. Highly specific role of hypocretin (orexin) neurons: differential activation as a function of diurnal phase, operant reinforcement vs. operant avoidance and light level. *Journal of Neuroscience* **31**, 15455-15467 (2011).

8. Harper,J.M. Gelineau's narcolepsy relieved by opiates. *Lancet*. **1**, 92 (1981).

9. Benbadis,S.R. Effective treatment of narcolepsy with codeine in a patient receiving hemodialysis. *Pharmacotherapy*: *The Journal of Human Pharmacology and Drug Therapy* **16**, 463-465 (1996).

10. Blouin,A.M. *et al*. Human hypocretin and melanin-concentrating hormone levels are linked to emotion and social interaction. *Nature Communications* **4:1547**, 1547 (2013).

11. Ripley,B., Fujiki,N., Okura,M., Mignot,E., & Nishino,S. Hypocretin levels in sporadic and familial cases of canine narcolepsy. *Neurobiol Dis*. **8**, 525-534 (2001).

12. Fry,J.M., Pressman,M.R., DiPhillipo,M.A., & Forst-Paulus,M. Treatment of narcolepsy with codeine. *Sleep*. **9**, 269-274 (1986).

13. Hockenhull,J., Murphy,K.G., & Paterson,S. An observed rise in +¦-hydroxybutyrate-associated deaths in London: Evidence to suggest a possible link with concomitant rise in chemsex. *Forensic Science International* **270**, 93-97 (2017).

14. Harris,G.C., Wimmer,M., & Aston-Jones,G. A role for lateral hypothalamic orexin neurons in reward seeking. *Nat*. **437**, 556-559 (2005).

15. Harris,G.C., Wimmer,M., Randall-Thompson,J.F., & Aston-Jones,G. Lateral hypothalamic orexin neurons are critically involved in learning to associate an environment with morphine reward. *Behav Brain Res*. **183**, 43-51 (2007).

16. Sharf,R., Sarhan,M., & DiLeone,R.J. Orexin mediates the expression of precipitated morphine withdrawal and concurrent activation of the nucleus accumbens shell. *Biol Psychiatry*. **64**, 175-183 (2008).

17. Narita,M. *et al.* Direct involvement of orexinergic systems in the activation of the mesolimbic dopamine pathway and related behaviors induced by morphine. *J Neurosci.* **26**, 398-405 (2006).

18. Scammell,T.E., Willie,J.T., Guilleminault,C., & Siegel,J.M. A consensus definition of cataplexy in mouse models of narcolepsy. *Sleep* **32**, 111-116 (2009).

19. Chemelli,R.M. *et al.* Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. *Cell* **98**, 437-451 (1999).

20. Morawska,M., Buchi,M.+., & Fendt,M. Narcoleptic episodes in orexin-deficient mice are increased by both attractive and aversive odors. *Behavioural Brain Research* **222**, 397-400 (2011).

21. Vassalli,A. *et al.* Electroencephalogram paroxysmal theta characterizes cataplexy in mice and children. *Brain* **136**, 1592-1608 (2013).