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Orexin gene transfer into the amygdala suppresses both spontaneous and emotion-induced cataplexy in orexin-knockout mice

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Abstract

Narcolepsy is a chronic sleep disorder linked to the loss of orexin-producing neurons in the hypothalamus. Cataplexy, a sudden loss of muscle tone during waking, is an important distinguishing symptom of narcolepsy and it is often triggered by strong emotions. The neural circuit underlying cataplexy attacks is not known, but is likely to involve the amygdala, a region implicated in regulating emotions. In mice models of narcolepsy, transfer of the orexin gene into surrogate neurons has been successful in ameliorating narcoleptic symptoms. However, it is not known whether this method also blocks cataplexy triggered by strong emotions. To examine this possibility, the gene encoding mouse prepro-orexin was transferred into amygdala neurons of orexin-knockout (KO) mice (rAAV-orexin; $n = 8$). Orexin-KO mice that did not receive gene transfer (no-rAAV; $n = 7$) or received only the reporter gene (rAAV-GFP; $n = 7$) served as controls. Three weeks later, the animal's sleep and behaviour were recorded at night (no-odour control night), followed by another recording at night in the presence of predator odour (odour night). Orexin-KO mice given the orexin gene transfer into surrogate amygdala neurons had significantly less spontaneous bouts of cataplexy, and predator odour did not induce cataplexy compared with control mice. Moreover, the mice with orexin gene transfer were awake more during the odour night. These results demonstrate that orexin gene transfer into amygdala neurons can suppress both spontaneous and emotion-induced cataplexy attacks in narcoleptic mice. It suggests that manipulating amygdala pathways is a potential strategy for treating cataplexy in narcolepsy.

Introduction

Narcolepsy is a chronic, life-long sleep disorder affecting almost 1 in 2000 Americans. The major symptoms of narcolepsy include excessive daytime sleepiness, cataplexy, sleep fragmentation and hypnogogic hallucinations. Because narcolepsy diminishes the patient's quality of life, many patients also develop various secondary psychological symptoms such as fatigue, stress, memory loss and depression (Aldrich, 1993; Mattarozzi *et al.*, 2008). Orexin, a peptide localized in a small group of neurons in the hypothalamus area, has been linked to narcolepsy because there is a profound loss of orexin-producing neurons in humans with narcolepsy (Nishino *et al.*, 2000; Peyron *et al.*, 2000; Thannickal *et al.*, 2000). Canines with narcolepsy have a mutation in the orexin-receptor-2 (Lin *et al.*, 1999), and mice with deletion of the orexin gene [orexin knockout (KO)] (Chemelli *et al.*, 1999) or with ablation of the orexin-producing neurons (Hara *et al.*, 2001) are valid animal models of the human disease.

Cataplexy, one of the symptoms of narcolepsy, is a sudden and transient attack of muscle weakness during waking, and is often triggered by strong emotions including both positive (e.g. laughter, humour) and negative (e.g. anger, fear or sudden surprise) emotions (Aldrich, 1993; Mattarozzi *et al.*, 2008). In narcoleptic mice and canines, cataplexy attacks can be triggered by emotions, such as food (Siegel *et al.*, 1991; Burgess *et al.*, 2013), wheel-running (Espana *et al.*, 2007) or predator odour (Morawska *et al.*, 2011). The mechanism responsible for emotion-induced cataplexy in humans and animals remains unclear, although the amygdala, a region important for emotion processing, might play a pivotal role in triggering emotion-induced cataplexy (Brabec *et al.*, 2011; Burgess *et al.*, 2013; Meletti *et al.*, 2015). Cataplexy-related neurons have been found in the amygdala of canines with narcolepsy (Gulyani *et al.*, 2002). Moreover, bilateral lesions of the amygdala reduce cataplexy induced by stimuli associated with 'positive emotions' (i.e. wheel running plus chocolate feeding) (Burgess *et al.*, 2013). Although amygdala lesions were successful in reducing cataplexy, there are likely to be

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significant adverse consequences on affect and cognition as a result of lesioning this critical brain structure.

On the other hand, orexin gene transfer into specific neurons in the brains of mice models of narcolepsy has successfully decreased spontaneous bouts of cataplexy (Liu *et al.*, 2008, 2011; Blanco-Centurion *et al.*, 2013). However, the effects of orexin gene transfer into the amygdala neurons have not been determined. Because of the link between emotion and cataplexy, we hypothesize that orexin gene transfer into surrogate amygdala neurons can rescue both spontaneous and emotion-induced cataplexy in narcoleptic mice. In the present study, predator odour (coyote urine) was used to trigger emotion-induced cataplexy in orexin-KO mice (Morawska *et al.*, 2011). We found that transfer of the orexin gene into surrogate neurons in the amygdala of orexin-KO mice blocked both spontaneous and emotion-induced cataplexy, and also increased waking. This is the first evidence that orexin gene transfer into a discrete neural circuit blocks spontaneous and emotion-induced cataplexy as well as increases waking in narcoleptic mice.

Materials and methods

Ethics statement

All manipulations done to the mice adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Medical University of South Carolina (protocol 3267) and the Ralph H. Johnson VA (protocol 537) Institutional Animal Care and Use Committee.

Construction and delivery of recombinant adeno-associated virus (rAAV) vector

Mouse prepro-orexin cDNA fragment (393 bp from Dr Yanagisawa's laboratory; University of Texas Southwestern Medical Center, Dallas, TX, USA) was cloned into an rAAV plasmid from Harvard Gene Therapy Initiative Laboratory (Boston, MA, USA), to form rAAV-orexin, in which orexin expression was driven by the cytomegalovirus promoter. The control virus, rAAV-GFP, was created by replacing orexin cDNA with green fluorescent protein (GFP) cDNA. Vector packaging and titration were done by the University of North Carolina Gene Therapy Initiative (final titre at $2.7–3.0 \times 10^{13}$ genomic copies/mL).

Orexin-KO mice

Homozygous orexin-KO mice derived from founders originally supplied by Dr Yanagisawa were used in this study. The mice have been backcrossed onto a C57BL/6J line for over 20 generations in our laboratory. Twenty-seven orexin-KO mice (male and female, 6–12 months old, 25–42 g) were randomly assigned to one of the following groups: rAAV-orexin (n = 10), rAAV-GFP (n = 10) and no-rAAV (n = 7).

Implanting sleep recording electrodes

Under deep anaesthesia (isoflurane 2.0%) viral vectors were microinjected into the central nucleus of the amygdala (1.2 mm posterior to bregma, 2.9 mm lateral and 4.6 mm ventral to brain surface; Paxinos & Franklin, 2013) using a stereotaxic instrument (Kopf, Tujunga, CA, USA). The experimental (rAAV-orexin) and control (rAAV-GFP) vectors were delivered in a volume of 500 nL on each side of the brain using a 2.0-μL Hamilton syringe coupled to a 33-gauge stainless steel injector (Plastics One, Roanoke, VA, USA). Injections were done gradually over 15 min. After the microinjection, the injector needle was left in place for 10 min and then withdrawn slowly. At this time the mice were also implanted with electrodes to record the electroencephalogram (EEG) and electromyogram (EMG) as described previously (Liu *et al.*, 2011). All electrode leads were then secured onto the skull with dental cement. After surgery the animals were housed singly in Plexiglas cages with food and water available *ad libitum*. The temperature in the sleep recording room was 25 °C and a 12-h light/dark cycle (lights on 07:00–19:00 h) was maintained.

Two weeks after surgery, the animals were connected to lightweight recording cables and allowed 1 week of adaptation. On day 21 the EEG/EMG signal and behaviour were continuously recorded during the 12-h lights-off period. Using a polygraph (AstroMed, Model 12; West Warwick, RI, USA) EEG/EMG signals were amplified, filtered (0.3–100 Hz for EEG; 100 Hz to 1 kHz for EMG), and then recorded onto a hard-disk with sleep data acquisition software (SleepSign; Kissei Comtec, Nagano, Japan). Behavioural data were also continuously recorded via night vision cameras. On day 23, 12 h of sleep and behaviour were again recorded in the presence of predator odour (coyote urine) during the 12-h lights-off period.

Delivery of predator odour

The predator odour test was on the night of day 23. In total, 1.0 mL coyote urine (www.Predatorpee.com, Bangor, ME, USA) was placed in a 5-mL glass vial filled with cotton. There were holes in the vial's plastic cap which allowed the mice to smell the predator odour. The vial was placed in the cage at the start of the animal's active period (lights-off cycle) for 12 h. EEG/EMG and video recording started simultaneously when the test began. We chose to deliver the odour during the active period (night) because cataplexy is most prevalent at night in narcoleptic mice (Liu *et al.*, 2011).

Identification of sleep–wake states, cataplexy

EEG, EMG and video recordings were scored manually with SleepSign software in 12-s epochs for wake, non-rapid eye movement (NREM) sleep, rapid eye movement (REM) sleep and cataplexy. Wakefulness was identified by the presence of desynchronized EEG and high EMG activity. NREM sleep consisted of high-amplitude slow waves together with a low EMG tone relative to waking. REM sleep was identified by the presence of regular EEG theta activity coupled with low EMG relative to slow wave sleep.

Cataplexy was identified by a sudden loss of muscle tone when the mice were awake. The following criteria were used to identify cataplexy: the episodes had to occur when the mouse was awake (≥36 s) and engaged in an active behaviour such as walking, running, grooming, eating or drinking; the episode had to last at least 12 s; during the episode, theta activity had to be present, and delta activity diminished; and there had to be loss of muscle tone based on EMG and video data (Scammell *et al.*, 2009; Liu *et al.*, 2011).

Immunostaining of transfected neurons

At the end of the study the mice were deeply anaesthetized with isoflurane (5%) and were perfused transcardially with 0.9% saline (5–10 mL) followed by 10% buffered formalin in 0.1 M phosphate-buffered saline (PBS, 50 mL). The brains were placed in 30% sucrose (0.1 M PBS) and allowed to equilibrate. The brains were cut on a cryostat (40-μm thick sections; coronal plane) and one-in-four series of sections were processed for visualization of orexin-A

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immunoreactivity. Briefly, the tissue was incubated overnight at room temperature in the goat anti-orexin-A antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1 : 10 000), then incubated for 1 h with a biotinylated secondary antibody (donkey anti-goat IgG, Jackson Immunoresearch Laboratories, West Grove, PA, USA; 1 : 500). The antibodies were visualized using the avidin/biotin/diaminobenzidine-nickel staining method (Vector Laboratories, Burlingame, CA, USA). Immunofluorescence was used to visualize two antigens. The free floating sections were incubated overnight in orexin-immunoreactive (goat anti-orexin-A; 1 : 500; Santa Cruz Biotechnology) in combination with vesicular GABA transporter (VGAT; rabbit anti-VGAT; 1 : 500; Millipore, Billerica, MA, USA), tyrosine hydroxylase (TH; rabbit anti-TH; 1 : 1000; Millipore), choline acetyltransferase (ChAT; rabbit anti-ChAT; 1 : 1000; Millipore) or serotonin (rat anti-serotonin; 1 : 1000; Millipore) immunoreactive (ir) neurons. For immunofluorescence the following secondary antibodies were used: donkey anti-goat-Alexa Fluor 488; donkey anti rabbit-Alexa Fluor 568 or donkey anti-rat Alexa Fluor 594 (1 : 500; Invitrogen, Carlsbad, CA, USA). The sections were then washed and mounted on to gelatin-coated slides. Photomicrographs were obtained with a Nikon confocal microscope.

Statistical analysis

Two-way repeated measures analysis of variance (ANOVA) with *post-hoc* tests (Bonferroni) compared the means among the three groups (Kirk, 1968). As there were unequal numbers of mice in each group (unbalanced design), the data were analysed using the general linear model (SPSS). Statistical significance was evaluated at the $P < 0.05$ (two-tailed) level.

Results

Distribution of orexin-A-ir surrogate neurons and terminals

We intentionally selected the orexin-KO mice model of narcolepsy for this study because the gene for prepro-orexin has been genetically deleted and there is no evidence of the peptide in the tissue in these mice (Chemelli *et al.*, 1999). We did not choose the orexin-ataxin-3 transgenic mice model of narcolepsy because not all of the orexin-producing neurons die from the accumulation of polyglutamine toxicity (Hara *et al.*, 2001). As such, in the orexin-ataxin-3 transgenic mice the orexin peptide is evident in degenerating nerve terminals and a few surviving orexin somata, which could confound the results.

None of the mice in this study had orexin-A-ir somata in the lateral hypothalamus, which confirms that these were orexin-KO mice. rAAV-orexin was inserted into the central nucleus of the amygdala (CeA) in ten orexin-KO mice. In two mice orexin-A-ir neurons were not observed in the injection site, perhaps because the injection cannula was clogged. In the remaining eight orexin-KO mice numerous orexin-A-ir somata and fibres were evident in the amygdala (six mice with bilateral distribution, two mice with unilateral distribution, Fig. 1). Most of the transfected somata were distributed in the CeA and basolateral amygdala (BLA) (Fig. 2A and B). Diffuse orexin-A-ir somata and terminals were also present in the basomedial amygdala and adjacent areas in some mice. Double immunostaining showed co-localization of orexin and γ-aminobutyric acid (GABA) in many transfected neurons (Fig. 2C–E). Distal orexin-A-ir terminals were found in the locus ceruleus (LC), ventral lateral periaqueductal grey (vlPAG) and the dorsal raphe (DR) (Fig. 2F–H). Orexin-A-ir was not found in the brains of control orexin-KO mice (no-rAAV and rAAV-GFP groups).

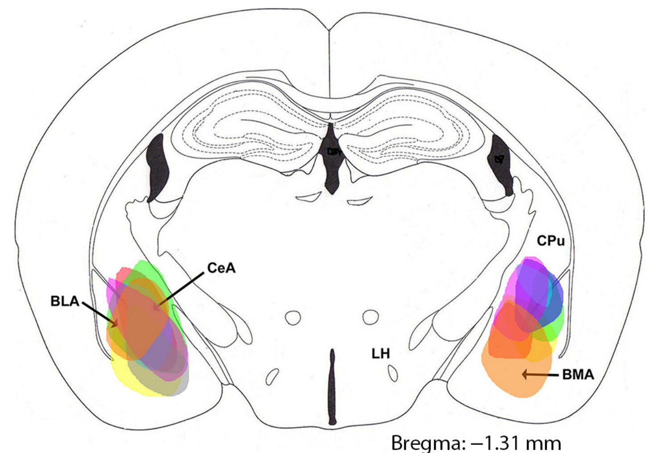

FIG. 1. Schematic distribution of orexin-A-immunoreactive somata in the amygdala 29 days after rAAV-orexin gene transfer. Each colour represents the distribution in one mouse (n = 8). The drawing represents a coronal section adapted from the mouse brain atlas (Paxinos & Franklin, 2013).

rAAV-GFP was injected into the amygdala in ten orexin-KO mice. In three mice GFP-positive somata were not evident in the injection site, perhaps because of clogged cannulae. In the remaining seven orexin-KO mice numerous GFP-positive somata were evident in the amygdala.

Effects on cataplexy

Table 1 summarizes the average number and length of bouts of cataplexy in the mice. There was a significant effect between groups ($F_{2,19}$ = 92.01, P = 0.001), exposure to odour ($F_{1,19}$ = 91.33, P = 0.001) and interaction ($F_{2,19}$ = 19.7, P = 0.001) on cataplexy number. Before exposure to the predator odour, there was no significant difference in the incidence of cataplexy between the two groups of control mice (no-rAAV vs. rAAV-GFP: t_{12} = 0.78, P = 0.93). However, these control mice had three-fold more spontaneous bouts of cataplexy compared with the orexin-KO mice given orexin gene transfer (no-rAAV vs. rAAV-orexin: t_{13} = 4.41, P = 0.001; rAAV-GFP vs. rAAV-orexin: t_{13} = 5.14, P = 0.001) (Table 1). When the mice were exposed to the predator odour, the control mice had twice as many bouts of cataplexy compared with the no-odour night (no-rAAV: t_{12} = 8.14, P = 0.001; rAAV-GFP: t_{12} = 7.56, P = 0.001; *post-hoc* vs. no-odour) and five times as many bouts compared with the mice with the orexin gene transfer (no-rAAV vs. rAAV-orexin: t_{13} = 12.05, P = 0.001; rAAV-GFP vs. rAAV-orexin: t_{13} = 12.20, P = 0.001) (Table 1). In the mice with the orexin gene transfer, exposure to the odour did not significantly increase the number of bouts of cataplexy (t_{14} = 0.54, P = 0.60, Table 1). Thus, predatory odour did not trigger cataplexy in mice given orexin gene transfer into the amygdala.

The percentage of time spent in cataplexy over the 12-h night period was also determined (Fig. 3) and the data analysed using a two-way repeated measures ANOVA (three groups by two-factor repeated measures – time in 3-h blocks nested within odour; Type split-plot factorial 3.42) (Kirk, 1968; p. 300). There was a significant between-group effect ($F_{2,19}$ = 87.95, P = 0.001) in that the orexin group had significantly less time in cataplexy compared with each of the two control groups (no-rAAV vs. rAAV-orexin: t_{13} = 7.82, P = 0.001; rAAV-GFP vs. rAAV-orexin: t_{13} = 7.36, P = 0.001). There was a significant effect of odour ($F_{1,19}$ = 44.65, P = 0.001) and a group by odour interaction effect ($F_{2,19}$ = 10.76,

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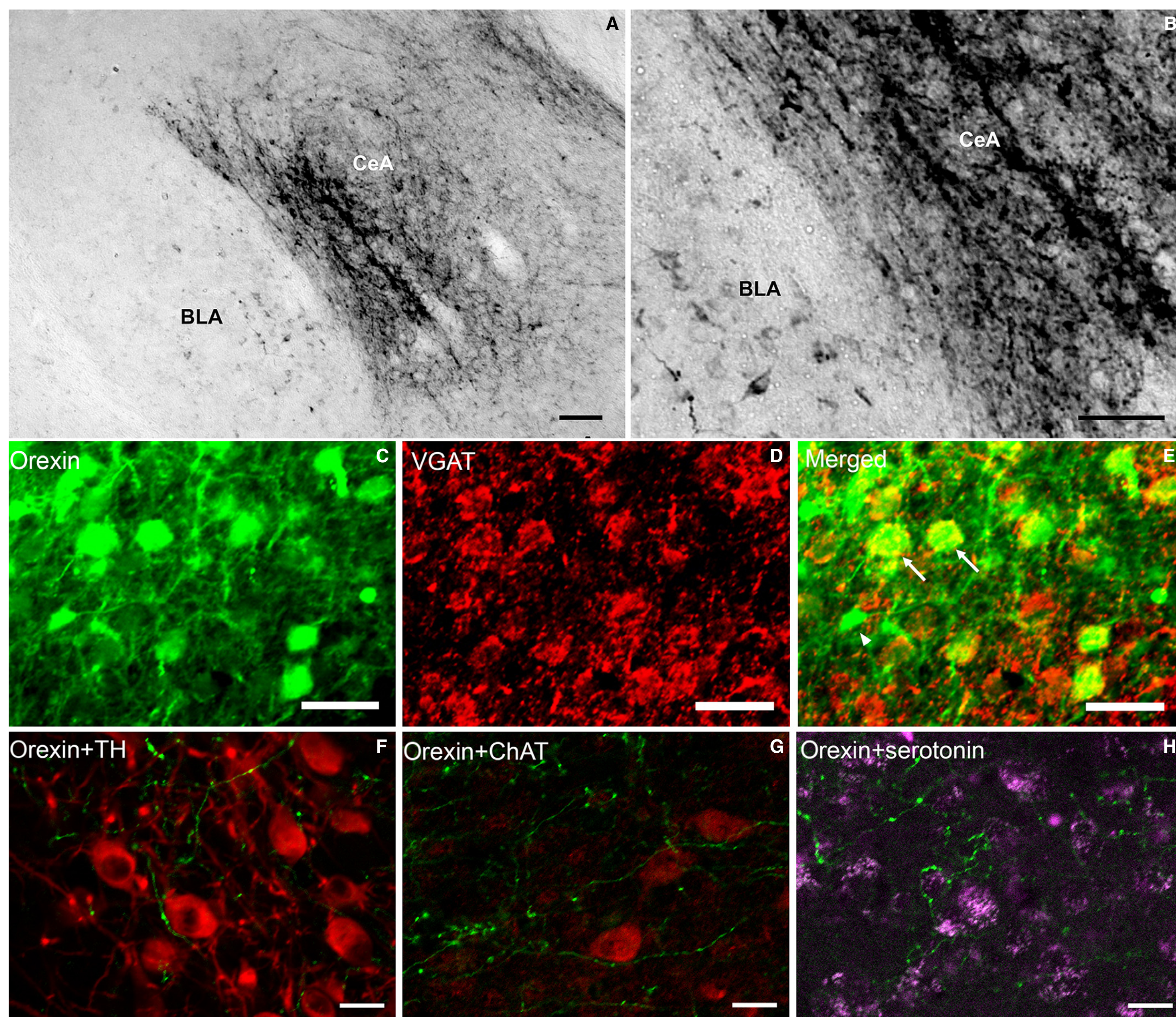

FIG. 2. Distribution of orexin-A-ir in the amygdala and pons of orexin-KO mice given rAAV-orexin in the amygdala. (A and B) Orexin-A-ir was distributed in somata and proximal dendrites of CeA and BLA cells. (C–E) Transfected prepro-orexin gene was expressed in GABA (arrows) and non-GABA (arrowhead) neurons in CeA. (F–H) Orexin-A-ir was also observed in distal terminals in the pons in close proximity to noradrenergic neurons of the LC (F), cholinergic neurons in the vlPAG (G) and serotonin neurons of the DR (H). Scale bar in A and B: 100 μm; C–H: 25 μm.

TABLE 1. Effect of orexin gene transfer on mean (±SEM) duration and number of cataplexy bouts

	Number		Duration (s)	
	No-odour night	Odour night	No-odour night	Odour night
no-rAAV (n = 7)	19.62 ± 0.89	43.79 ± 1.32#	21.57 ± 1.87	20.47 ± 1.69
rAAV-GFP (n = 7)	21.71 ± 0.92	44.14 ± 1.10#	24.78 ± 1.85	22.97 ± 1.99
rAAV-orexin (n = 8)	6.40 ± 0.46*	8.00 ± 0.36**	20.48 ± 1.85	21.97 ± 1.66

The data were analysed with a repeated-measures ANOVA (GLM model) with *post-hoc* comparisons (Bonferroni). During the no-odour night, the control groups (no-rAAV and rAAV-GFP) had three times more spontaneous bouts of cataplexy compared with the orexin-KO mice with orexin gene transfer (*P = 0.001). Upon exposure to predator odour (coyote urine) the control groups had twice as many bouts of cataplexy compared with the no-odour night (#P = 0.001) and five times as many bouts as the mice given orexin gene transfer (**P = 0.001). In orexin-KO mice the predator odour did not significantly trigger cataplexy. There was no significant change in average length of cataplexy bouts among all three groups.

P = 0.001). There was a significant interaction effect (group × time × odour) ($F_{6,57}$ = 4.23, P = 0.001) with the two control groups having more percentage cataplexy in the first 3 h of the light-off cycle compared to the last quarter of the night (Fig. 3A and B). This is consistent with published data (Chemelli *et al.*, 1999) that in orexin-KO mice cataplexy attacks dominate during the

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beginning of the night cycle. Indeed, for the night cycle, before exposure to the odour control mice spent significantly more percentage of time in cataplexy compared with the mice with the orexin gene transfer (no-rAAV vs. rAAV-orexin: $t_{13} = 4.38$, $P = 0.001$; rAAV-GFP vs. rAAV-orexin: $t_{13} = 4.40$, $P = 0.001$) (Fig. 3D). In the two control groups exposure to the odour significantly increased percentage cataplexy compared with the no-odour night (Fig. 3A, B and D). When first exposed to the predator odour the percentage of time spent in cataplexy in the rAAV-orexin group did not increase, and across the 12-h night period of odour exposure there was no significant difference in percentage cataplexy when compared with the no-odour control night ($t_7 = 0.32$, $P = 0.75$; Fig. 3C and D). Thus, orexin gene transfer blocked emotion-induced cataplexy.

Effects on waking and NREM/REM sleep

There was no significant difference in percentage waking during the no-odour control night between the three groups. Thus, orexin gene transfer into the amygdala did not increase spontaneous levels of waking. However, when mice were exposed to the predator odour, orexin gene transfer into the amygdala made the mice significantly more awake compared with the no-odour night ($t_{14} = 2.51$, $P = 0.021$). The no-rAAV and rAAV-GFP groups had slight increases in waking but these were not significant (Fig. 4A). Total NREM sleep time in the rAAV-orexin group was significantly reduced in response to predator odour ($t_{14} = 2.38$, $P = 0.028$, Fig. 4B). There was no significant change in total REM sleep time between control night and predator odour night in all three groups (Fig. 4C).

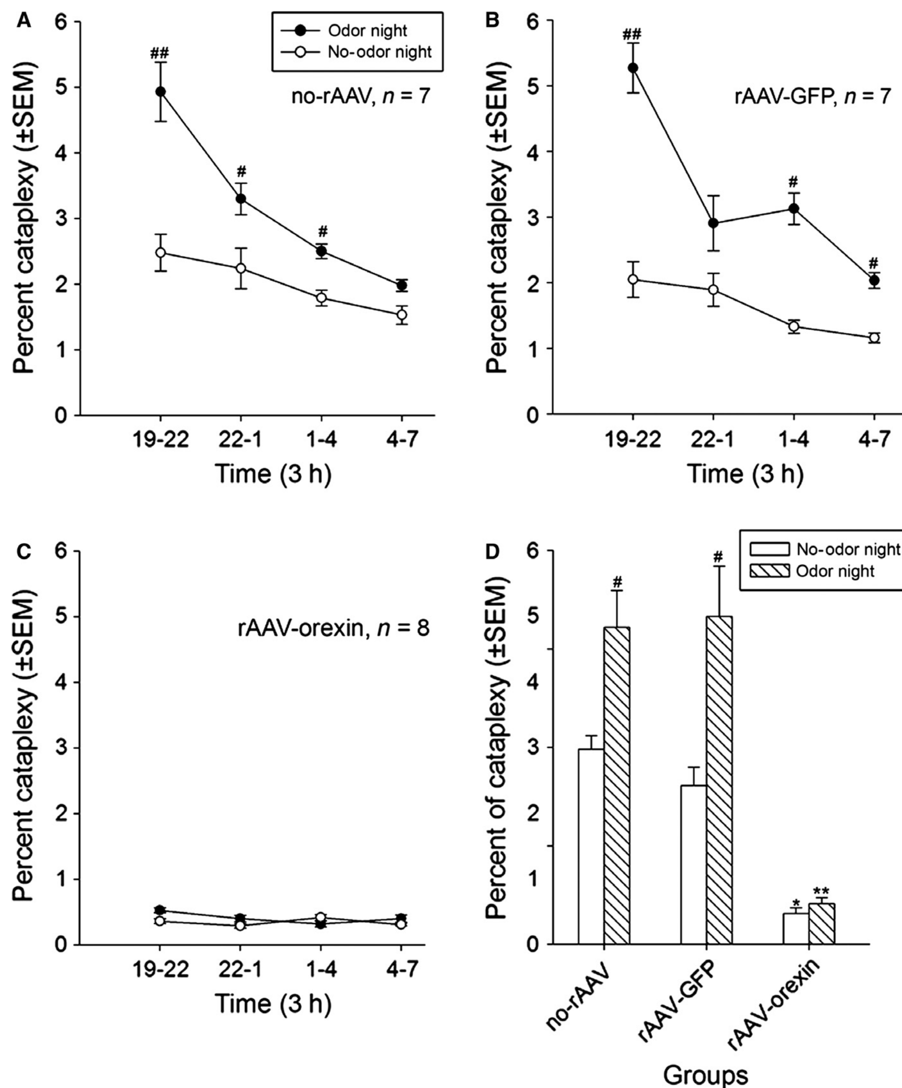

FIG. 3. Effects of orexin gene transfer into amygdala on per cent (±SEM) cataplexy during the 12-h light-off period (night). (A–C) Summarize the time-course of per cent cataplexy across the 12-h night period, with each data point representing a 3-h average (±SEM). Data were analysed with repeated measures ANOVA (SPF-3.42 design; GLM model). (D) Summarizes the data over the 12-h night period. Cataplexy was determined on the basis of both EEG/EMG and video behavioural data. The control groups are orexin-KO mice given no virus (no-rAAV) or given the reporter gene, GFP (rAAV-GFP). The experimental group is orexin-KO mice given orexin gene transfer into the amygdala (rAAV-orexin). The experimental group spent significantly less time in spontaneous cataplexy, and exposure to predator odour did not increase cataplexy in these mice. On the other hand, predator odour (coyote urine) increased per cent cataplexy in both control groups. #$P < 0.05$, vs. no-odour control night; ##$P = 0.001$, vs. no-odour control night; *$P = 0.001$, vs. no-rAAV and rAAV-GFP groups at no-odour control night; **$P = 0.001$, vs. no-rAAV and rAAV-GFP groups at odour night.

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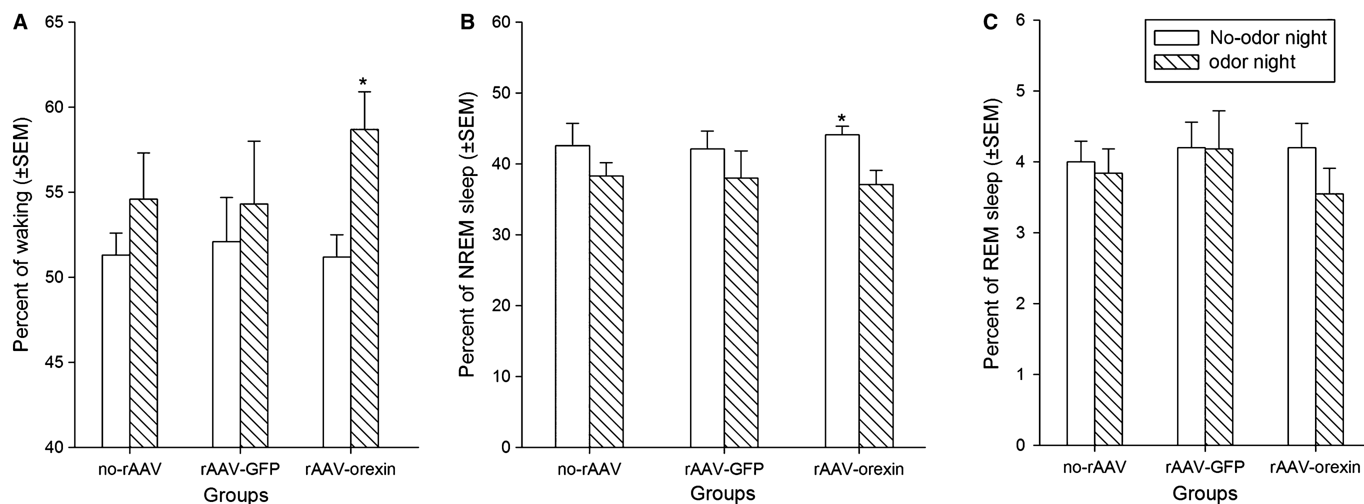

FIG. 4. Effects of orexin gene transfer in amygdala on per cent (±SEM) waking, NREM sleep and REM sleep during the 12-h light-off period (night). When exposed to predator odour, the orexin-KO mice with orexin gene transfer in amygdala had a significant increase in waking (A, $*P = 0.021$ vs. no-odour control night) and a significant decrease in NREM sleep (B, $*P = 0.028$ vs. no-odour control night). The other two groups also showed an increase in waking but this was not significant. Exposure to predator odour did not change per cent REM sleep (C).

Discussion

The primary result of this study was that orexin gene transfer into surrogate neurons of the amygdala (mostly CeA and BLA) suppressed not only spontaneous but also emotion-induced cataplexy behaviour in the orexin-KO mice. Orexin-KO mice exhibit a behavioural phenotype that is strikingly similar to human narcolepsy patients (Chemelli *et al.*, 1999). Like human narcolepsy, cataplexy in orexin-KO mice is triggered by strong positive (e.g. chocolate reward) and negative emotions (e.g. predator odour) (Morawska *et al.*, 2011; Burgess *et al.*, 2013). Previously, in orexin-KO mice spontaneous bouts of cataplexy were rescued by ectopic expression of the prepro-orexin gene in all cells (Willie *et al.*, 2011). However, such a strategy does not identify a pathway involved in specific behaviours. On the other hand, site-directed orexin gene transfer limits the number of surrogate neurons expressing the orexin gene and also identifies a potential neural circuit. Our previous studies (Liu *et al.*, 2008, 2011; Blanco-Centurion *et al.*, 2013) and other data (Hasegawa *et al.*, 2014) determined that spontaneous bouts of cataplexy can be rescued by orexin gene transfer into specific brain neurons. However, those studies did not assess emotion-induced cataplexy. The amygdala was selected as the primary site because of the evidence linking it to cataplexy (Gulyani *et al.*, 2002; Burgess *et al.*, 2013).

The behaviour of the mice during the predator odour night was similar to a previous study that also utilized predator odour to induce cataplexy (Morawska *et al.*, 2011). Upon odour delivery mice displayed various behavioural symptoms of mild fear responses including multiple direct examinations of the bottle (source of the odour), biting and burying the bottle, or running around to get away from the odour. During the no-odour night spontaneous bouts of cataplexy occurred when mice were eating, walking or grooming. However, during the odour night cataplexy in the two control groups was mostly triggered by odour-induced behaviours such as bottle biting or running (no-rAAV and rAAV-GFP). In the two control groups, the increase in cataplexy with activity such as running is consistent with a previous study (Espana *et al.*, 2007) in which wheel-running behaviour increased cataplexy. Mice in the rAAV-orexin group exhibited similar responses during the odour night, but it did not induce cataplexy. Unfortunately, we did not directly measure locomotor activity levels, but the rAAV-orexin group was awake more in response to the predator odour (Fig. 4A). However, the increased waking did not induce cataplexy, indicating that emotion-induced cataplexy was blocked with orexin gene insertion into amygdala neurons.

The amygdala plays an important role in emotion processing (Phelps & LeDoux, 2005; Kim *et al.*, 2011). Predator odour-induced fear responses have been found to trigger c-Fos expression in the central (CeA) and basolateral (BLA) nucleus of the amygdala (Butler *et al.*, 2011). Some of these activated neurons might regulate muscle tone by projecting directly to the pons. In wild-type mice, pontine neurons receive not only GABA inhibitory control from the amygdala, but also orexin excitatory innervations from the lateral hypothalamus. These innervations maintain muscle tone during emotional stress, allowing the animal to escape. There is anatomical support for such a circuit. For instance, there are dense reciprocal connections between orexin neurons and the amygdala (Marcus *et al.*, 2001; Cluderay *et al.*, 2002). Orexin excites both GABA neurons and corticotropin-releasing factor (CRF) neurons in the CeA via orexin receptors (Bisetti *et al.*, 2006). The amygdala projects to pontine structures controlling muscle tone such as the vlPAG/lateral pontine tegmentum and DR (Boissard *et al.*, 2003; Fung *et al.*, 2011).

When orexin signalling is absent, as in narcolepsy, the balance between inhibitory and excitatory control on muscle tone is weakened, resulting in muscle atonia during waking. By re-introducing orexin into the amygdala–pons circuit, the balance was restored and cataplexy was significantly reduced (Fig. 5). We suggest that in the present study during emotional distress the amygdala GABA neurons also released orexin, in addition to GABA, at pontine target sites. However, the presence of orexin stabilized muscle tone and blocked both spontaneous and emotion-induced cataplexy.

We found that some of the transfected orexin-expressing neurons in amygdala were GABAergic and orexin-A-ir terminals were observed in pons in close proximity to neurons in the vlPAG, LC and DR, which are the critical downstream targets of amygdala for regulating muscle tone (Fig. 2). We also noted that many non-GABA neurons were also transfected and there are some excitatory projections from amygdala such as CRF projection, which might also participate in cataplexy behaviour during emotions. Indeed, CRF neurons in CeA are involved in fear response (Butler *et al.*,

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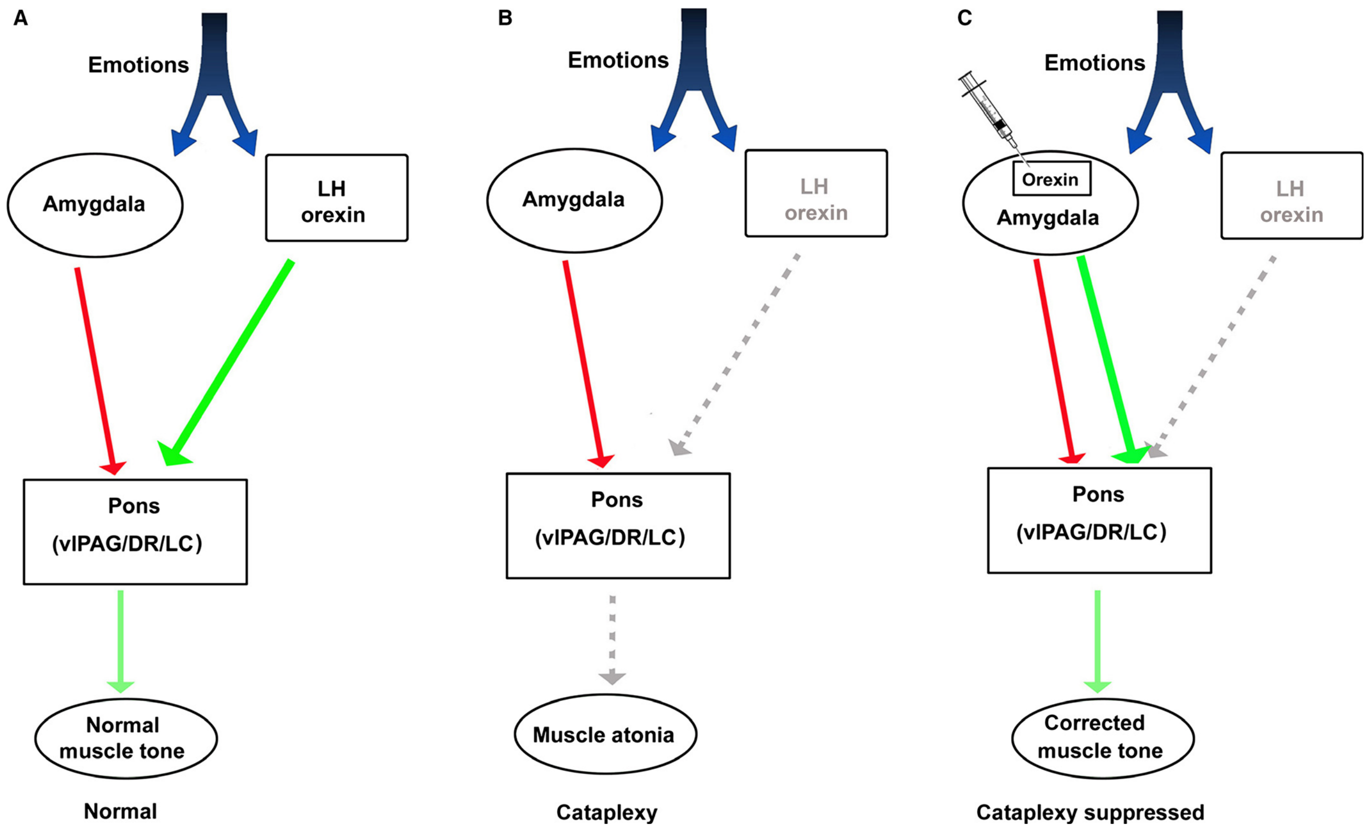

FIG. 5. A neural circuit model summarizes how orexin gene transfer into amygdala may rescue cataplexy. Evidence from many studies indicates that muscle tone is regulated by the activity of pontine neurons (Fraigne *et al.*, 2015). During emotions the orexin excitatory signal from the lateral hypothalamus neutralizes the inhibitory signal from the amygdala and maintains the activity of pontine neurons (A). In narcoleptic mice the orexin excitatory signal from the lateral hypothalamus is absent. Therefore, the inhibitory signal from the amygdala overwhelms and inhibits pontine neurons controlling muscle tone, resulting in muscle atonia during waking (i.e. cataplexy) (B). When the orexin gene is inserted into surrogate neurons in the amygdala, the excitatory orexin signal is re-introduced into the amygdala–pons circuit that maintains muscle tone, and cataplexy is suppressed (C).

2011). As the rAAV vector used in this study transferred the orexin gene into many neurons in the amygdala the specific phenotype of the projection responsible for regulating cataplexy attacks cannot be identified. Phenotype-specific or projection-specific orexin gene transfer should be done in future studies to delineate the neural circuit regulating cataplexy.

Our use of the orexin gene transfer method has been very useful in identifying surrogate neurons that ameliorate cataplexy. Areas in the brain where orexin gene transfer has decreased spontaneous bouts of cataplexy in orexin-KO and orexin/ataxin-3 mice models of narcolepsy include the lateral hypothalamus, the zona incerta and the dorsolateral pons (Liu *et al.*, 2008, 2011; Blanco-Centurion *et al.*, 2013). We have now determined that orexin can be placed into amygdala neurons and it blocks both spontaneous and emotion-induced cataplexy. These studies indicate that to decrease cataplexy orexin must be placed in surrogate neurons that project directly to the pontine neuronal circuitry regulating muscle tone. Orexin gene transfer into the striatum has no effect on cataplexy (Liu *et al.*, 2011) even though the striatum regulates motor behaviour. This is because the orexin is in local striatal interneurons which do not directly release orexin onto pontine targets. We have also determined that along with the direct innervation of the pons the surrogate neurons must also be active in waking. The activity in waking would release orexin to stabilize the motor circuit. For instance, cataplexy is not decreased when orexin is placed in melanin-concentrating hormone (MCH)-containing neurons of the posterior hypothalamus (Liu *et al.*, 2011). The MCH neurons innervate the pons (Yoon & Lee, 2013) but they are silent during waking (Hassani *et al.*, 2009). As such, when cataplexy is triggered these neurons are silent, indicating that orexin is not released from these transfected MCH neurons.

In conclusion, we have provided the first evidence that orexin gene transfer into surrogate neurons in the amygdala suppresses both spontaneous and emotion-induced cataplexy attacks. Further studies will identify the phenotype of amygdala neurons and their downstream targets regulating muscle tone during emotions. The gene transfer approach can serve as a tool to repair neural circuits regulating specific behaviours, such as muscle tone in narcolepsy.

Conflict of interest

The authors declare no conflicts of interest, financial or otherwise.

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Abbreviations

ANOVA, analysis of variance; BLA, basolateral amygdala; CeA, central nucleus of amygdala; ChAT, choline acetyltransferase; CRF, corticotropin releasing factor; DR, dorsal raphe; EEG, electroencephalogram; EMG, electromyogram; GFP, green fluorescent protein; ir, immunoreactive; KO, knockout; LC, locus coeruleus; MCH, melanin-concentrating hormone; NREM,

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non-rapid eye movement; rAAV, recombinant adeno-associated virus; REM, rapid eye movement; TH, tyrosine hydroxylase; VGAT, vesicular GABA transporter; vlPAG, ventrolateral periaqueductal grey.

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