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Research Report

Intraventricular orexin-A improves arousal and early EEG entropy in rats after cardiac arrest

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ABSTRACT

The recovery of arousal after cardiac arrest (CA) is associated with evolution from electroencephalographic (EEG) burst-suppression to continuous activity. Orexin-A elicits arousal EEG during anesthetic burst-suppression. We hypothesized that orexin-A would improve arousal and EEG entropy after CA. Eighteen Wistar rats were subjected to 7-minute asphyxial CA and resuscitation. Rats were divided into treatment ($n=9$) and control ($n=9$) groups. Twenty minutes after resuscitation, the treatment group received 0.1 mL of 1 nM orexin-A intraventricularly, while controls received saline. EEG was quantified using Information Quantity (IQ), a measure of entropy validated for detection of burst-suppression and arousal patterns. IQ values range from 0 to 1.0. Arousal was quantified using the neurological deficit scale (NDS). The ischemic neuronal fraction of hippocampus CA1 and cortex was histologically determined. Baseline and post-resuscitation characteristics were similar between the groups. The NDS score (mean \pm SD) at 4 h was higher in the orexin-A group compared to controls (57.3 ± 5.8 vs. 40.7 ± 5.9 , $p<0.02$), but scores were similar at 72 h. Burst frequency was similar in both groups but the orexin-A group demonstrated higher IQ values compared to controls beginning within 10 min. IQ values remained significantly higher in the orexin-A group for the first 120 min ($p=0.008$) and subsequently converged. The ischemic neuronal fraction was similar between groups in cortex ($p=0.54$) and hippocampus CA1 ($p=0.14$). In rats resuscitated from CA, orexin-A transiently increased arousal and EEG entropy without worsening ischemic neuronal injury. The role of orexin-A in recovery of arousal after CA deserves further investigation.

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1. Introduction

The CDC estimates that 460,000 cardiac arrests (CA) occur annually in the United States, with survival rates of 5–8% in

most centers (Rosamond et al., 2008). The majority of survivors are initially comatose and disorders of consciousness represent the leading cause of chronic disability (Bedell et al., 1983; Berek et al., 1997; Vaagenes et al., 1997). Coma is presumably

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Abbreviations: EEG, electroencephalogram; CA, cardiac arrest; IQ, Information Quantity; qEEG, quantitative electroencephalogram; NDS, neurological deficit scale; H&E, hematoxylin and eosin; ROSC, return of spontaneous circulation; HDS, histological damage score; MAP, mean arterial blood pressure

Table 1 – Baseline characteristics of orexin and control groups

	Control (n=9)	Orexin-A (n=9)	p-value
Weight (g)	343±25	367±27	0.094
Time to CA (s)	124±26	114±27	0.446
Time to ROSC (s)	31.6±6.6	31.4±6.9	0.971
Baseline IQ	0.569±0.030	0.597±0.035	0.118
Time to bursting (min)	16.1±0.9	14.6±2.0	0.073
pH post-ROSC	7.29±0.08	7.32±0.04	0.303
PCO ₂ post-ROSC (mmHg)	34.0±5.2	35.0±1.7	0.593
HCO ₃ post-ROSC (mmol/L)	15.9±2.6	17.9±1.7	0.080
MAP 20 min post-ROSC (mmHg)	123.2±24.5	125.1±23.3	0.875
MAP 40 min post-ROSC (mmHg)	110.5±16.2	112.6±21.5	0.539
MAP 60 min post-ROSC (mmHg)	116.8±20.2	121.6±16.6	0.428

Time to CA is the interval between onset of asphyxia and pulse pressure <10 mmHg; time to ROSC is the interval between onset of sternal compressions and return of spontaneous circulation (ROSC); baseline IQ is the no-normalized IQ value prior to asphyxia; time to bursting is the interval between ROSC and onset of EEG burst-suppression; arterial blood gas values were determined 10 min after ROSC; MAP is mean arterial blood pressure.

due to diffuse cortical injury and selective injury to subcortical areas such as the thalamus (Longstreth, 2001; Auer and Sutherland, 2002).

Ischemic neuronal injury suppresses synaptic transmission, which is measured by changes in EEG (Holmes et al., 1983). In humans, EEG is suppressed within seconds of CA (Clute and Levy, 1990; Losasso et al., 1992; Moss and Rockoff, 1980) and recovers via an initial periodic bursting pattern that precedes restitution of continuous activity (Jorgensen and Malchow-Moller, 1981a). This transition from burst-suppression to continuous EEG precedes arousal by hours to days (Jorgensen and Malchow-Moller, 1981b). Persistent burst-suppression or failure to regain continuous EEG activity results in permanent unresponsiveness (Jorgensen and Malchow-Moller, 1981b; Scollo-Lavizzari and Bassetti, 1987). The same pattern of EEG recovery after CA has also been described in rats (Geocadin et al., 2002; Luft et al., 2002; Rosen et al., 1984), dogs (Gurvitch et al., 1972; Lin et al., 1977; Lind et al., 1975), piglets (Sherman et al., 1999), and monkeys (Myers and Yamaguchi, 1977).

In our previous work, we determined that the evolution of EEG activity after CA can be quantified using an entropy-based measure called Information Quantity (IQ) (Shin et al., 2006; Jia et al., 2006, 2008). IQ tracks recovery of EEG from low entropy states (burst-suppression) to high entropy states (desynchronized, reactive EEG). We have previously shown that rapid increase in IQ after CA is associated with faster arousal and better neurological outcomes (Jia et al., 2006, 2008).

Physiologic processes involved in resolution of EEG burst-suppression, return of continuous EEG activity, and arousal have not been studied after CA.

The hypothalamic neuropeptide orexin-A has recently emerged as a potent mediator of arousal via widespread

synapses in the thalamus, cortex, and ascending brainstem networks (Eggermann et al., 2001; Marcus et al., 2001; Govidaiah and Cox, 2006; Bayer et al., 2002). Orexin deficiency has been demonstrated after global ischemia and orexin receptors are upregulated in ischemic brain (Nakamachi et al., 2005; Irving et al., 2002). Recently, intraventricular injection of orexin-A in rats with anesthetic-induced burst-suppression resulted in rapid desynchronization of EEG activity and resolution of burst-suppression (Yasuda et al., 2003; Sato-Suzuki et al., 2002; Dong et al., 2006). Orexin administration also shortens anesthetic time (Kushikata et al., 2003) whereas, inhibition of orexinergic signaling delays anesthetic emergence (Kelz et al., 2008).

Based on these observations, we hypothesized that intraventricular injection of orexin-A in rats resuscitated from CA would improve early arousal and increase EEG entropy, as quantified by IQ.

2. Results

A total of 18 rats were subjected to 7-minute asphyxial CA and resuscitation with continuous monitoring of EEG. Rats were randomly assigned to receive either 0.1 mL of 1 nM orexin-A (n=9) by intraventricular infusion or an identical volume of normal saline (n=9) 20 min after ROSC. As shown in Table 1, baseline characteristics of the 2 groups were not significantly different prior to drug delivery. There were trends for lower serum bicarbonate concentration and longer interval prior to initiation of burst-suppression in control animals but these were not statistically significant. Mean arterial blood pressure (MAP) measurements were similar just prior to orexin-A or saline infusion (20 min post-ROSC) and there were also no significant differences in MAP between the two groups after drug infusion (Table 1).

Recovery was monitored using serial NDS scores calculated by an examiner who was masked to the treatment assignment at 4, 24, 36, and 72 h after ROSC. As shown in Table 2, the mean NDS score was significantly higher at 4 and 24 h after ROSC in the orexin-A group compared to controls. NDS scores were similar between groups at 48 and 72 h. Data were reported as mean±SD at the first 2 time points because the distribution of values was normal. The other points had a left-skewed distribution of data, so NDS values were reported as median and interquartile range. The 10-point difference between groups at the 4-hour NDS measurement largely reflected a greater proportion of

Table 2 – Neurological deficit scale (NDS) in orexin-A and control groups

	Control	Orexin	p-value
4-hour NDS	44.6±6.0	54.1±5.3	0.004
24-hour NDS	62.3±6.1	70.3±3.1	0.004
48-hour NDS	70 (65–74)	74 (72–74)	0.128
72-hour NDS	72 (68–74)	74 (72–74)	0.180
4 and 24 h: mean±SD, 48 and 72 h: median (interquartile range).			

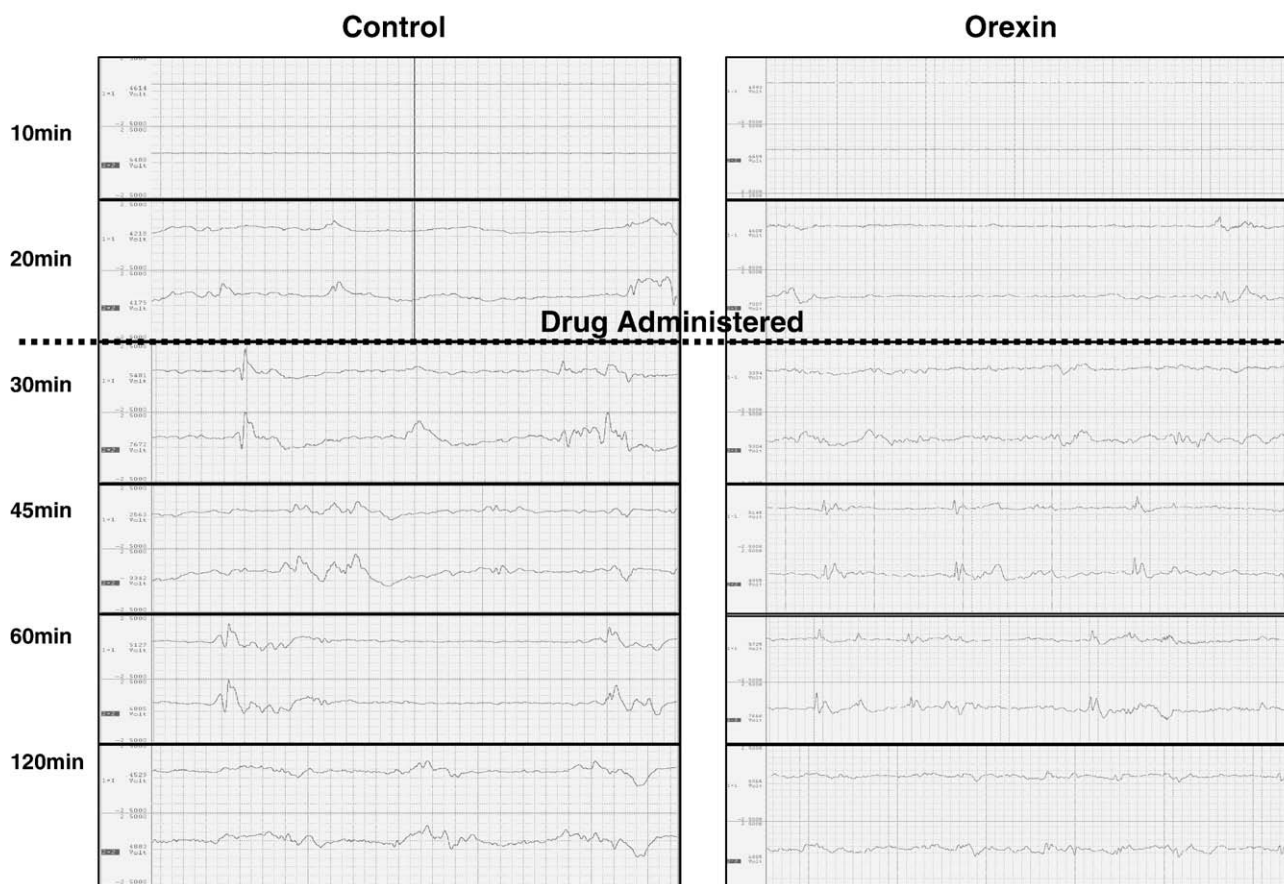


Fig. 1 – Raw EEG recordings from representative orexin-A and control rats at serial intervals after return of spontaneous circulation (ROSC). Labeled intervals are time after ROSC, with orexin-A or saline administered at 20 min. Epochs between 30 and 60 min demonstrate burst-suppression, while the 120-minute epoch shows return of continuous EEG activity. Subjective EEG interpretation did not reveal obvious differences between the two groups.

animals with spontaneous eye opening, respiration, and withdrawal from noxious stimulation in the orexin-A group.

EEG was analyzed off-line for quality assurance and removal of artifact. Qualitative assessment of raw recordings from representative animals did not reveal major differences in EEG signals between the orexin-A and control groups (Fig. 1). Burst counts were performed by an examiner masked to treatment assignments by averaging manually counted EEG bursts over 5-minute periods during standard intervals after ROSC. As shown in Table 3, there was no significant difference in burst frequency between the groups before drug administration or at any point afterward.

EEG entropy was then calculated using the IQ algorithm. As shown in Fig. 2, there was a significantly greater increase in IQ 10 min after orexin-A infusion compared to animals receiving placebo. IQ values were significantly higher in orexin-A-treated rats compared to controls during the first 120 min after ROSC ($p=0.008$), after which IQ values in the 2 groups converged. The sharpest increase in IQ occurred within 10 min of orexin infusion. Subsequently, IQ values remained similar between groups (Table 4).

Animals were continuously observed for clinical seizure activity during the first 6 h after ROSC and during daytime hours for the remaining 72 h prior to sacrifice. EEG was also reviewed for electrical seizures during the recording periods. No clinical or EEG seizures were observed in any of the rats during the 72-hour experiment.

All rats survived resuscitation from CA and were sacrificed 72 h after ROSC. Histological evaluation verified correct placement of cannulae in the lateral ventricle. Quantification of ischemic neuronal injury was undertaken in the hippocampus CA1 sector and primary sensory cortex (Fig. 3). Ischemic neurons had similar frequency in the orexin-A and control animals. HDS scores in the cortex were 0.278 ± 0.087 in controls and 0.250 ± 0.077 in the orexin group

Table 3 – Burst frequency during serial periods after ROSC

Burst frequency	Control	Orexin	p-value
20 min	42.7 ± 22.4	52.2 ± 25.0	0.488
30 min	71.9 ± 31.5	94.3 ± 41.4	0.290
40 min	85.6 ± 27.9	105.3 ± 46.2	0.362
50 min	86.9 ± 34.4	89.3 ± 55.9	0.924

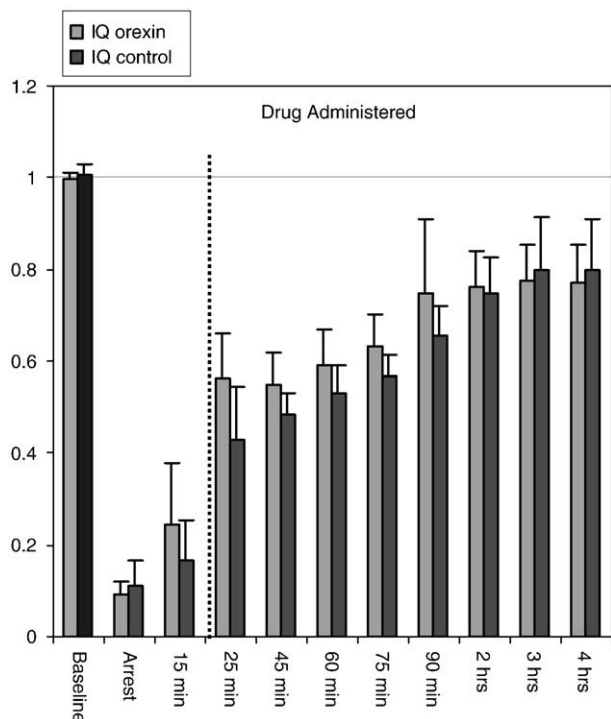


Fig. 2 – Mean normalized IQ values with standard deviation bars in orexin-A and control groups at baseline, during CA, and at serial intervals after return of spontaneous circulation (ROSC). The Y-axis depicts IQ values normalized to baseline (range 0–1) and the X-axis depicts time. Using a General Linear Model to analyze for repeated measures, IQ was significantly higher in the orexin-A group compared to controls ($p=0.008$) during the first 120 min. IQ values subsequently converged in the two groups.

($p=0.54$). In the hippocampus CA1, HDS scores were 0.252 ± 0.056 in controls and 0.199 ± 0.069 in the orexin-A group ($p=0.14$).

3. Discussion

Recovery from CA proceeds through an intermediate phase of coma associated with EEG burst-suppression (Geocadin et al., 2000b, Jia et al., 2006; Scollo-Lavizzari and Bassetti, 1987). In humans, recovery of continuous EEG activity often precedes arousal by hours to days (Jorgensen and Malchow-Moller, 1981b). Orexin-A is an endogenous neuropeptide that stimulates and maintains arousal from sleep (Sakurai, 2007) and pharmacological coma induced by barbiturates and isoflurane (Dong et al., 2006; Kushikata et al., 2003). Interference with orexinergic transmission delays emergence from anesthesia (Kelz et al., 2008). Intraventricular infusion of orexin-A in rats rapidly produces arousal EEG patterns during isoflurane-induced burst-suppression (Kushikata et al., 2003; Sato-Suzuki et al., 2002; Yasuda et al., 2003). We studied the effect of intraventricular infusion of orexin-A on early arousal and EEG entropy in comatose rats resuscitated from CA.

This experiment demonstrated a modest but functionally significant increase in arousal behavior 4 h after CA in rats treated with a single dose of intraventricular orexin-A compared to controls. The increase in NDS was maintained 24 h after CA, after which NDS was similar between the control and orexin-A groups. Although the increase in NDS at 4 h among animals treated with orexin-A was relatively modest, this degree of change reflects potentially important functional differences. In particular, animals treated with orexin-A scored higher in categories of spontaneous eye opening, respiration, and withdrawal from noxious stimulation at 4 h. At 24 h, animals in the orexin-A group scored higher in the complex behavioral categories of the NDS. Our previous studies demonstrated an association between early increase in EEG entropy and resolution of burst-suppression with higher NDS at 72 h (Jia et al., 2006, 2008). In the present experiment, however, pharmacological enhancement of early entropy with orexin-A did not produce sustained effects on 72-hour NDS.

The NDS is patterned on the coma exam currently employed for clinical evaluation and includes measures of eye opening, cranial nerve reflexes, response to noxious stimuli, and interaction with the environment. Some aspects of the NDS may be subjective, such as eye opening. We attempted to limit potential bias by using an examiner who was masked to the treatment group assignment. We performed the first NDS evaluation 4 h after CA in order to ensure that all animals could be extubated and decannulated for performance of the entire NDS battery. Because of residual pharmacological paralysis, the NDS could not be performed soon after orexin-A infusion, which limits our ability to comment on the timing of arousal. However, it is important to note that the resolution of burst-suppression in human survivors of CA typically precedes clinical signs of arousal by hours to days (Jorgensen and Malchow-Moller, 1981a,b). In addition, studies of orexin-A infusion during anesthetic burst-suppression have demonstrated a delayed effect on arousal (Kushikata et al., 2003).

Using HDS, we did not demonstrate an increase in cortical or hippocampal ischemic neuronal injury in animals treated with orexin-A. This is an important finding, because widespread neuronal activation induced by orexin-A shortly after a hypoxic-ischemic insult could potentially worsen ischemic injury through increased release of extracellular glutamate (John et al., 2003; Huang et al., 2006). In addition, NDS scores at 72 h were similar between the 2 groups suggesting that a single dose of orexin-A does not worsen brain injury after global ischemia.

Unlike studies of orexin-A in anesthetic burst-suppression (Yasuda et al., 2003; Dong et al., 2006), we did not demonstrate

Table 4 – Serial IQ values in orexin-A and control groups

	Control	Orexin	p-value
4-hour IQ	0.798 ± 0.110	0.759 ± 0.086	$p=0.442$
24-hour IQ	0.924 ± 0.075	0.915 ± 0.113	$p=0.862$
48-hour IQ	0.907 ± 0.051	0.929 ± 0.101	$p=0.634$
72-hour IQ	0.885 ± 0.085	0.893 ± 0.111	$p=0.887$
Mean \pm SD.			

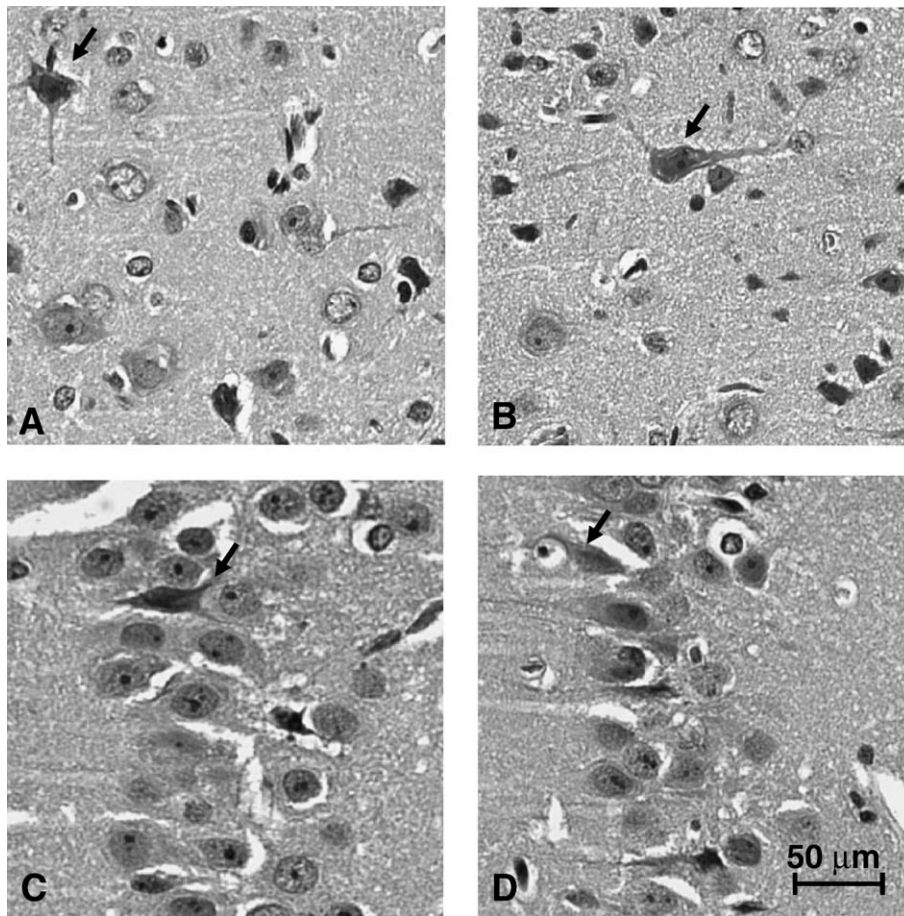


Fig. 3 – H&E stained photomicrographs (40× resolution) showing similar ischemic neuronal injury in layer IV of the sensory cortex in control (A) and orexin-A (B) animals and hippocampus CA1 in control (C) and orexin-A (D) animals. The histological damage score (HDS) was 0.278 ± 0.087 in controls and 0.250 ± 0.077 in the orexin-A group in cortex ($p=0.54$) and 0.252 ± 0.056 in controls and 0.199 ± 0.069 in the orexin-A group in hippocampus CA1 ($p=0.14$).

a qualitative change in burst frequency or restitution of continuous EEG activity after orexin-A infusion. Using IQ to quantify EEG entropy, however, we demonstrated a significant increase in IQ values within 10 min of orexin infusion, which was maintained for the first 2 h after ROSC. This duration of action is consistent with the reported half-life of orexin-A after a single intraventricular dose (Yoshida et al., 2003). The discrepancy between IQ and burst frequency results is likely explained by limitations in qualitative measures of burst-suppression like burst frequency. In particular, simple methods of burst counting are insensitive to changes in burst duration and complexity that are better measured by entropy (Sherman et al., 1999; Geocadin et al., 2002).

Although they require confirmation and further validation, these findings have interesting implications for research into the mechanisms and treatment of burst-suppression coma in CA survivors. In humans who emerge from coma after resuscitation from CA arrest, transient burst-suppression is a frequently observed EEG pattern prior to arousal (Jorgensen and Malchow-Moller, 1981b; Scollo-Lavizzari and Bassetti, 1987). Persistence of burst-suppression for >24 h after ROSC is strongly associated with poor prognosis (Wijdicks et al., 2006; Scollo-Lavizzari and Bassetti,

1987) and contributes to decisions to limit life-sustaining therapies (Geocadin et al., 2006). Despite its prognostic significance in this setting, very little is understood about the physiology of burst-suppression and the mechanisms of restitution of continuous EEG activity.

Because of its known role in initiating arousal through widespread activation of thalamic, cortical, and brainstem centers and its ability to desynchronize EEG rhythms (Eggermann et al., 2001; Marcus et al., 2001; Govidaiah and Cox, 2006; Bayer et al., 2002), orexin-A is an interesting candidate neuropeptide to study in recovery after CA. Orexin-A selectively depolarizes and excites nonspecific thalamocortical projection neurons in the centromedial and paraventricular nuclei of the thalamus, but has no effect on specific sensory relay neurons (Bayer et al., 2002; Govidaiah and Cox, 2006). It also increases depolarization rates of prefrontal cortical neurons which have been implicated in attention and arousal (Lambe et al., 2005). Widespread cortical activation produced by nonspecific thalamocortical projections and prefrontal cortical networks are integral to both maintenance of arousal and desynchronization of EEG signal during burst-suppression (Dringenberg and Olmstead, 2003; Dong et al., 2006). Greater EEG desynchronization by activation of these thalamocortical

and intracortical networks can then be quantified using entropy measures like IQ.

Some important limitations to this study must be acknowledged. First, the treatment group sizes were modest, which may have contributed to statistically insignificant differences in some post-CA parameters, ie. time to ROSC and serum HCO₃ concentration (Table 1). We did not monitor cerebral blood flow during the experiment, so we could not determine whether increased arousal may have been caused by augmentation of cerebral perfusion in the orexin-A group. Similar to other studies of intraventricular administration of orexin-A (Yasuda et al., 2003), however, we did not find any significant increase in systemic blood pressure compared to controls. These data argue against a passive increase in cerebral perfusion by augmentation of systemic blood pressure in the setting of impaired cerebral autoregulation.

In addition, a single dosage of orexin-A was studied, so a dose–response relationship could not be established. The 1 nM concentration of orexin-A was chosen based on other publications showing effective resolution of anesthetic burst-suppression with intraventricular infusion (Yasuda et al., 2003; Dong et al., 2006). The results of this study require verification and extension to include both continuous infusion of orexin-A and multiple dosing tiers in order to test whether sustained effects and a dose–response relationship exist.

Finally, intraventricular administration limits the potential for translation of these findings to humans. The data on CNS penetrance of peripherally administered orexin-A are conflicting. In rodents, orexin-A rapidly diffuses across the blood brain barrier at a low concentration (Kastin and Akerstrom, 1999; John et al., 2003). In narcoleptic dogs, appreciable CSF concentrations were only achieved with high peripheral doses (Fujiki et al., 2003), although others have reported behavioral effects with peripheral administration (John et al., 2000). CNS penetrance of orexin-A has not been determined in humans and the impact of CA on disruption of the blood brain barrier is another important variable. Regardless, high peripheral doses of orexin-A would likely be required to achieve CSF concentrations similar to intraventricular administration. As with the potential treatment of narcolepsy, optimism for drug development may be greater for orexin analogs rather than the endogenous neuropeptide (Fujiki et al., 2003).

The translational implications of these findings are unclear, at present. Given the transient increase in arousal and EEG activation, we do not anticipate that orexin-A will have a therapeutic application for improving long term outcomes after CA. If administration of orexin-A has similar effects in humans, however, there could be a benefit to shortening the duration of coma in patients who are capable of recovering. In addition, CA survivors who demonstrate an increase in EEG entropy in response to orexin-A may be more likely to emerge from coma. Earlier knowledge of the potential to recover arousal after CA may have important implications for decisions about continuation or limitation of supportive care.

In summary, we show that a single intraventricular dose of orexin-A administered post-injury in a rodent model of asphyxial CA rapidly improved arousal and increased EEG entropy without worsening neuronal injury. Considering that

coma and disorders of arousal continue to be a major problem after resuscitation, orexin-A deserves more intensive study.

4. Experimental procedures

Eighteen adult male Wistar rats (300–350 g, Charles River, Wilmington MA, USA) subjected to 7-minute asphyxial CA were randomly assigned to the treatment or placebo groups. The experimental protocol was approved by the Johns Hopkins Animal Care and Use Committee.

4.1. Experimental asphyxia-CA model

The asphyxial CA and cardiopulmonary resuscitation (CPR) model used previously validated protocols (Jia et al., 2006, 2008). In brief, epidural screw electrodes (Plastics One, Roanoke, VA) were implanted 7 days prior to CA. An intraventricular guide cannula (0.4 mm inner diameter, 0.7 mm outer diameter, Plastics One, Roanoke, VA) was stereotactically inserted into the right lateral ventricle via a burr hole using standard coordinates (Paxinos and Watson, 1997). The guide cannula was secured with dental cement and a dust cover (Plastics One, Roanoke, VA) was placed.

On the day of CA, rats were endotracheally intubated by laryngoscopy and ventilated with 1.0% halothane in N₂/O₂ (50%/50%). The femoral artery and vein were cannulated to monitor mean arterial pressure (MAP), sample arterial blood gases (ABG), and administer saline and drugs. Five minutes of baseline recording with halothane was followed by a 3-minute washout to ensure no significant residual effect of halothane on EEG. CA was initiated via asphyxia with paralysis by vecuronium 2 mg/kg IV and cessation of mechanical ventilation for 7 min. CA time was defined by pulse pressure <10 mmHg. Cardiopulmonary resuscitation (CPR) was performed with epinephrine 5 mcg/kg IV, sodium bicarbonate 1 mmol/kg IV, and sternal compressions (200/min) until return of spontaneous circulation (ROSC). ABGs were obtained 4 times per animal: at baseline, and 10-, 20-, and 40-min after ROSC. Sedative agents were not used after CA in order to avoid confounding influences on EEG. Temperature was continuously maintained between 36.0 and 37.0 °C via a warming blanket and monitored by a rectal sensor. Extubation, removal of vascular cannulae, and surgical closure were performed when the rat was breathing and moving spontaneously.

4.2. Neurological evaluation

Arousal and neurological recovery were quantified using the Neurological Deficit Scale (NDS). As previously described (Jia et al., 2006, 2008), the NDS (Table 5) was patterned after the coma examination in humans, including measures of arousal, cranial nerve reflexes, and motor behavior. The NDS was determined 4 h after ROSC and then repeated at 24-, 48-, and 72-h. The examination was performed by a trained examiner masked to treatment group and the primary outcome measure of this experiment was defined as the 4-hour NDS score. This measurement was chosen to standardize timing of the evaluation because the majority of rats could safely undergo extubation and decannulation within 4 h of ROSC.

Table 5 – Neurological deficit scale (NDS)

A) Arousal
Alerting: normal [10]/stuporous [5]/comatose [0] Eye opening: open spontaneously [3]/open to pain [1]/absent [0] Spontaneous respiration: normal [6]/abnormal [3]/absent [0] Total score: 19
B) Brainstem function
Olfaction: present [3]/absent [0] Vision: blinks to threat [3]/absent [0] Pupillary light reflex: present [3]/absent [0] Corneal reflex: present [3]/absent [0] Startle reflex: present [3]/absent [0] Whisker stimulation: present [3]/absent [0] Swallowing: present [3]/absent [0] Total score: 21
C) Motor assessment
Strength: normal [3]/weak movement [1]/no movement [0] Total score: 6 (each side tested and scored separately)
D) Sensory assessment
Pain: brisk withdrawal [3]/weak withdrawal [1]/no movement [0] Total score: 6 (each side tested and scored separately)
E) Motor behavior
Gait coordination: normal [3]/abnormal [1]/absent [0] Balance beam walking: normal [3]/abnormal [1]/absent [0] Total score: 6
F) Behavior
Righting reflex: normal [3]/abnormal [1]/absent [0] Negative geotaxis: normal [3]/abnormal [1]/absent [0] Visual placing: normal [3]/abnormal [1]/absent [0] Turning alley: normal [3]/abnormal [1]/absent [0] Total score: 12
G) Seizures
Seizures: no seizure [10]/focal seizure [5]/generalized seizure [0] Total score: 10
Best outcome = 80, worst outcome = 0.

4.3. EEG recording and quantitative EEG analysis

Two channels of EEG were recorded in the right and left parietal areas. The signal was digitized using the data acquisition package CODAS (DATAQ Instruments INC., Akron OH). Sampling frequency of 250 Hz and 12 bits A/D conversion was used. Serial 30-minute EEG recordings were then performed at 24-, 48-, and 72-h after ROSC in each group. Signals were examined for noise, both in time domain using WinDaq software (DATAQ Instruments INC., Akron OH), and in frequency spectrum using Matlab (MathWorks, Natick, MA) and epochs contaminated by artifact were excluded from analysis. Manual counts were also performed to determine burst frequency in serial 5-minute periods.

Quantitative analysis of EEG was undertaken using Information Quantity (IQ). As previously described (Shin et al., 2006; Jia et al., 2006), the EEG waveform was divided into a series of

windows of equal length. Wavelet coefficients were then computed using a discrete wavelet transform. The distribution of the wavelet coefficients within a window was then determined by constructing a histogram. Using the frequency of wavelet coefficients in each bin of the histogram, the information was calculated using the formula for Shannon entropy. The entropy value of wavelet coefficients for a single window of EEG is called the Information Quantity (IQ).

To quantify how entropy evolves over time, IQ was averaged over several intervals. To compare IQ between multiple rats, the IQ averages were normalized to a baseline mean, which is the average IQ for the time window preceding CA. Normalized IQ values range from 1.0 (normal) to 0 (isoelectric), such that higher values reflect greater EEG entropy relative to baseline. We calculated mean IQ values over 5-minute intervals every 15 min from ROSC up to 90 min then hourly between 2 and 4 h. Mean IQ values were then calculated over 30-minute intervals at 24, 48, and 72 h after ROSC.

4.4. Histological evaluation

Animals were sacrificed via transcardiac perfusion with 4% PFA solution. Brains were immersed in 4% PFA for 24 h then transferred to 15% sucrose and 30% PBS. Paraffin-embedded brains were then sliced in 10 μ m sections, placed on slides, and stained with H&E. Histological damage score (HDS) was then calculated in the hippocampus CA1 and sensory cortical layers IV and V using an Olympus BX51 microscope (Olympus, Center Valley, PA) at 40 \times magnification and a standard rat brain atlas (Paxinos and Watson, 1997). Ischemic neurons were identified using standard criteria (Eke et al., 1990; Geocadin et al., 2000a): pyknosis, karyorrhexis, karyolysis, and cytoplasmic changes. Slides were reviewed by an investigator who was masked to treatment assignments. StereoInvestigator software (version 5.00, MicroBrightField Inc., Colchester, VT) was used to define a set of random, non-overlapping microscope fields. The area fractionator function was applied for 250 \times 250 μ m grids for cortex and 150 \times 150 μ m grids for CA1. These data were used to calculate a percentage of injury ((injured cells/total number of counted cells) \times 100) representing the HDS. Brains were also examined to verify placement of the cannula in the lateral ventricle.

4.5. Treatment groups

Animals were randomly assigned to receive either a single 10 μ L dose of 1 nM orexin-A (Phoenix Pharmaceuticals) via intraventricular infusion over 1 min or an equal volume of saline. Orexin-A dilutions were made on the day of the experiment and allowed to warm up to room temperature prior to injection. Saline placebo was maintained at room temperature prior to injection. The infusion of orexin-A or placebo began 20 min after ROSC through an introducer cannula custom fit to the guide cannula (Plastics One, Roanoke, VA) via a microinfusion pump (CMA, Solna, Sweden). The dose and concentration of orexin-A were chosen based on previous studies of orexin-A in anesthetic-induced burst-suppression (Yasuda et al., 2003; Dong et al., 2006). The timing of infusion was chosen because most rats begin to demonstrate EEG burst-suppression 15–20 min after ROSC (Jia et al., 2006, 2008; Geocadin et al., 2000b).

4.6. Statistical methods

Statistical analysis was performed using a computerized statistical package (Statistics Program for the Social Sciences version 16, Chicago IL). The Kolmogorov–Smirnov test was applied to evaluate normalcy of distribution. Group values that were parametric were reported as mean \pm SD and non-parametric variables were reported as median (interquartile range). Univariate analysis was performed for parametric data with Student's *t*-test for continuous variables, chi-square for categorical variables, and the least significant differences test for multiple comparisons. Non-parametric analysis of variance was used to test for differences in rank order NDS as a repeated measure. Alpha <0.05 was selected to consider differences significant.

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