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Apnea promotes glutamate-induced excitotoxicity in hippocampal neurons

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Abstract

Patients with obstructive sleep apnea (OSA) exhibit hippocampal damage and cognitive deficits. To determine the effect of apnea on the synaptic transmission in the hippocampus, we performed electrophysiological studies in an in vivo guinea pig model of OSA. Specifically, we determined the cornu ammonis region 1 (CA1) field excitatory postsynaptic potential (fEPSP) response to cornu ammonis region 3 (CA3) stimulation and examined the presynaptic mechanisms underlying the changes in the fEPSP. Single episodes of apnea resulted in a maximal potentiation of the fEPSPs at one to three minutes after the termination of each episode of apnea. The mean amplitude and slope of the post-apneic fEPSP was significantly larger compared with the pre-apneic control. These changes were accompanied by a significant decrease in the paired-pulse facilitation ratio during the post-apneic period compared with the pre-apneic control. The N-methyl-D-aspartate (NMDA) glutamate receptor antagonist MK-801, when applied locally to the CA1 recording site by pressure ejection, blocked the apnea-induced potentiation of the fEPSP. In the experimental animals that were subjected to extended periods of recurrent apnea, CA1 neurons exhibited positive immunoreactivity for fragmented DNA strands, which indicates apoptotic cell death. The present results demonstrate that apnea-induced potentiation of the hippocampal CA1 fEPSP is mediated by an NMDA receptor mechanism. We therefore conclude that recurrent apnea produces abnormally high levels of glutamate that results in the apoptosis of CA1 neurons. We hypothesize that this damage is reflected by the cognitive deficits that are commonly observed in patients with breathing disorders such as OSA.

Keywords

CA1; fEPSP; paired-pulse facilitation; hypoxia; excitotoxicity; guinea pig

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1. Introduction

Hippocampal neurons in humans and animals have been shown to be highly vulnerable to hypoxic/metabolic insults (Pulsinelli et al., 1982; Zola-Morgan et al., 1986; Cherubini et al., 1989; Ng et al., 1989). In rats, in conjunction with chronic episodes of hypoxia, there is a marked apoptosis in the cornu ammonis 1 (CA1) region of the hippocampus, although cornu ammonis 3 (CA3) neurons are generally unaffected (Gozal et al., 2001). In humans, high-resolution magnetic resonance imaging studies have revealed a significant loss of gray matter in the CA1 region of the hippocampus in patients with obstructive sleep apnea (OSA) (Macey et al., 2002).

Cellular damage to the CA1 region likely contributes to neuropsychological impairment including cognitive dysfunction among OSA patients (Engleman et al., 2000), since neural circuits in the hippocampus are important in learning and memory (Sweatt, 2004). For example, one of the daytime sequelae of OSA is impaired cognitive performance (Engleman et al., 2000; Sateia, 2003). Furthermore, there exists a significant correlation between disease severity (as indicated by polysomnographic indices of apnea-hypopnea index, arousal index, and minimum oxygen saturation) and cognitive deficits in sleep apnea/hypopnea syndrome (Engleman et al., 2000). In animal studies, brief periods of hypoxia (Sun et al., 2002) as well as chronic episodes of hypoxia (Gozal et al., 2001) are commonly associated with deficits in spatial learning and memory which most likely are hippocampal-dependent.

Experiments over the past two decades have established that hypoxia-related cell death involves calcium overloading as well as glutamate excitotoxicity (Choi, 1988a,b). Anatomically, there is a high convergence of glutamatergic inputs from Schaffer collaterals onto CA1 pyramidal neurons (Johnston and Amaral, 1998) which contain high densities of N-methyl-D-aspartate (NMDA) receptors (Monaghan and Cotman, 1985). Previous *in vivo* hippocampal microdialysis studies have revealed that cerebral ischemia evokes excessive vesicular release of glutamate (Benveniste et al., 1984; Mitani et al., 1994). This enhanced release of glutamate is consistent with the finding that an NMDA receptor-dependent hyperexcitability of CA1 neurons occurs after transiently exposing hippocampal slices to hypoxic media (Hori et al., 1991). In the hippocampus, it has been demonstrated that an increase in the presynaptic release of glutamate is accompanied by a reduction in the paired-pulse facilitation (PPF) ratio of CA3-CA1 synapses (Andersen, 1960; Alger and Teyler, 1976; Creager et al., 1980; Hess et al., 1987). Thus, the preceding data suggest that hypoxia-induced changes in synaptic transmission in CA1 involve an excessive release of glutamate.

Previously, *in vitro* studies have provided fundamental data dealing with hypoxia-related changes in synaptic processes. The paradigms that were employed involved prolonged periods of hypoxia or even anoxia lacking a hypercapnic component (Coelho et al., 2000; Sebastiao et al., 2001), which is different from the intermittent blood gas abnormalities (hypoxemia and hypercapnia) that occur in patients with OSA (Beebe and Gozal, 2002; Tafil-Klawe et al., 2004). To circumvent the limitations of previous paradigms, we developed an *in vivo* preparation to examine, electrophysiologically and pharmacologically, the hypothesis that apnea episodes result in an abnormally enhanced synaptic efficacy of CA1 pyramidal neurons due to the increased presynaptic release of glutamate. An increase in presynaptic release was confirmed electrophysiologically by a reduced PPF ratio of CA1 neurons following each episode of apnea. Our hypothesis that excitotoxicity occurs due to an excess of glutamate in conjunction with recurrent episodes of apnea was validated by immunostaining techniques that revealed apoptotic changes in CA1 neurons. The present findings may explain, at least in part, the hippocampal-dependent cognitive deficits that occur in OSA patients.

2. Results

2.1. Input-output functions of CA3-CA1 synapses

To study the monosynaptic transmission of CA3-CA1 synapses, *in vivo*, we examined single stimulus evoked field excitatory postsynaptic potentials (fEPSPs) in the dendritic region (stratum radiatum) of CA1 pyramidal neurons. Optimization and stability of recording conditions were ascertained, in individual experiments, through mapping studies by varying, systematically, the location of the stimulus and/or the recording electrodes. Under optimal recording conditions, low-intensity stimulation of the CA3 Schaffer collateral/commissural fibers evoked a fast-rising, negative fEPSP with maximal amplitude in the mid-distal (apical) dendritic region (i.e., stratum radiatum) remote from the somatic layer (stratum pyramidale) of CA1 region. In four animals, the stimulus-response relationship (input-output curve) was systematically constructed using graded stimulus intensities. Input-output curves measured by the fEPSP peak amplitude (Fig. 1A) and maximal slope parameters (Fig. 1B), are shown for one representative experiment; patterns of these curves from the remaining three experiments were qualitatively similar. Generally, the averaged CA1 fEPSP responses exhibited a pattern of incremental change with graded stimuli to the CA3 afferents, except along the plateau of the curve, wherein fEPSPs were unchanged in spite of further increases in the stimulus intensity (Fig. 1C, bottommost traces at 40 and 50 μ A intensities). Based upon individual input-output curves, an optimal baseline fEPSP that was free from contamination of population spike potentials and also situated on the linear slope of the input-output curve was present typically at 30–50% of the maximal amplitude of the fEPSP. Therefore, we utilized a control fEPSP the amplitude of which was approximately 50% of the maximum, prior to the onset of apnea, for all experiments involving post-apneic changes in fEPSPs and paired-pulse facilitation of CA1 neurons.

2.2. Apnea-induced potentiation of the CA1 fEPSP

In 12 animals, temporal changes in CA1 fEPSP responses to single-pulse stimulation of CA3 afferents were quantified (by the mean amplitude and slope parameters, $n = 12$ consecutive traces) during control (pre-apneic), apneic, and post-apneic conditions. From a total of 24 sets of data during control-apnea-recovery conditions, the mean apneic episode was found to last for 82.9 ± 2.2 sec (range = 60 to 110 sec) and the mean SpO_2 level was $54.3 \pm 0.6\%$ (range = 50 to 60%). During apnea, statistically non-significant decreases in the fEPSP amplitude and slope were observed (not shown). However, major increases in both the fEPSP amplitude and slope occurred at one to three minutes after the termination of apnea (Fig. 2A and B). Substantial potentiation remained up to five minutes post-apnea; for most animals complete recovery occurred at approximately 15 minutes post-apnea. In three animals that exhibited a persistent enhancement in fEPSPs beyond the 15-minute time point, the insignificant (but measurable) potentiation continued to decay at a slower rate until 30 minutes post-apnea. The representative temporal build-up and decay of the potentiation of fEPSPs, in one experiment, are illustrated in Fig. 2C–E, wherein full recovery of the fEPSP to baseline (pre-apneic) value was observed at 27 minutes post-apnea (Fig. 2E).

2.3. Apnea-induced attenuation of the paired-pulse facilitation

To assess apnea-induced changes in the paired-pulse plasticity of CA3-CA1 synapses that would support a presynaptic role of glutamate-mediated potentiation of fEPSPs, we analyzed, in a total of 14 trials pooled from a group of eight animals, changes in paired-pulse facilitation (PPF) ratios during pre- versus post-apneic conditions. With the paired-pulse paradigm, two consecutive stimuli delivered at an inter-stimulus interval of between 30–40 ms elicited a pair of fEPSPs with the second fEPSP amplitude larger than the first, resulting in the PPF of fEPSPs. Figure 3 illustrates the temporal changes in the PPF ratios, as measured by both fEPSP amplitudes (Fig. 3A) and slopes (Fig. 3B), in conjunction with

apnea (duration and SpO₂ levels were the same as those described above). Both measures of fEPSPs indicated that apnea strongly attenuated (i.e., with statistical significance; Fig. 3A and B) the PPF ratio by both amplitude and slope measurements for up to five minutes post-apnea. Pooled data indicated that the time course of the development (with a peak reduction in PPF ratio at two to three minutes post-apnea) and that the decay of the attenuation of PPF ratios resembled closely that of the apnea-induced potentiation of the fEPSP (Fig. 2A, B). Representative data of apnea-induced attenuation of the PPF ratio, from one animal, is displayed in Fig. 3C and D. The maximal reduction of the PPF ratio occurred at three minutes post-apnea. Compared with the pre-apneic control (PPF ratios = 1.38 and 1.63 for amplitude and slope values, respectively; Fig. 3C), a marked reduction in the PPF ratio occurred following the termination of apnea (PPF ratios = 1.02 and 1.24 for amplitude and slope values, respectively; Fig. 3D).

2.4. Effect of MK-801 on apnea-induced synaptic changes

The N-methyl-D-aspartate (NMDA) receptor antagonist MK-801 was applied locally and systemically, in separate animals, to determine the receptors that are involved in the apnea-induced potentiation of the CA1 fEPSP. In order to determine the direct effect of MK-801 on neurons in the CA1 region of the hippocampus, the drug was applied locally, by pressure ejection, via a drug-filled glass micropipette affixed to the recording microelectrode. Figure 4B depicts a single episode of apnea that evoked a marked (28.9%) potentiation of the amplitude of the fEPSP compared with the pre-apneic control prior to the application of MK-801 (Fig. 4A). Potentiation of the fEPSP by apnea was completely blocked within one minute after the intra-hippocampal administration of MK-801 (100 μ M in saline, Fig. 4C) by pressure ejection (10 psi, 10 sec). Thereafter, there was a lack of potentiation of the fEPSP throughout the period of apnea as shown in Fig. 2A. In another animal, MK-801 was injected intravenously in order to assess the global effects of the blockade of NMDA receptors, since the drug passes through the blood-brain barrier (Kemp et al., 1987). After the injection of MK-801 (2mg/Kg in saline, i.v.) 15 minutes prior to the induction of apnea, there was no change in the amplitude of the fEPSP and apnea was no longer able to produce any potentiation of the fEPSP (data not shown).

2.5. Recurrent apnea results in apoptotic damage to CA1 neurons

To address the hypothesized excitotoxic effect of cumulative increases in the release of glutamate (and hence the over-activation of NMDA receptors) associated with recurrent episodes of apnea, we evaluated potential morphological signs of neuronal damage in three experimental animals that underwent three hours of recurrent apnea; these data were compared with that obtained from a control group of two animals with normal ventilation and normoxic conditions. The results of one representative animal from each of the experimental and the control groups are shown in Fig. 5. Immunostaining with a monoclonal antibody against single-stranded DNA revealed that positively-labeled neurons were present in the CA1 region in the experimental animal. Most labeled neurons were localized in the stratum pyramidale where pyramidal CA1 neurons predominate; less neuronal labeling was observed in strata that were adjacent to the CA1 pyramidal neurons (Fig. 5B). In contrast, the control animal was devoid of labeled neurons in CA1 (Fig. 5A).

3. Discussion

3.1. In vivo guinea pig model of apnea

Obstructive sleep apnea (OSA) is a multi-factorial disease involving recurrent processes of oxygen deprivation (hypoxia), hypercapnia, increased cellular production of reactive oxygen species (oxidative stress-related injury to neurons), increased cardiovascular (sympathetic) as well as ventilatory responses (for reviews, see Beebe and Gozal, 2002; Lavie, 2003;

Narkiewicz and Somers, 2003; O'Driscoll and Morrell, 2005; Dincer and O'Neill, 2006). Numerous studies have utilized in vitro slice and cell culture preparations to address the cellular effects of hypoxia by exposing neurons to extremely low to 0% oxygen for prolonged periods equal to or greater than 30 minutes (Fernandez-Lopez et al., 2005; Hoffmann et al., 2006), making it difficult to correlate the resultant data with clinical findings in sleep apnea patients. An in vivo rodent model of intermittent hypoxia described by Gozal and coworkers (Gozal et al., 2001) provides correlative behavioral and anatomical consequences due to chronic intermittent hypoxia. In this model, the ambient carbon dioxide is maintained at normocapnic levels (0.03%), whereas the arterial pCO₂ is hypocapnic during the daily twelve hours treatment of intermittent hypoxia (ibid.). Patients with OSA are documented to exhibit various end-tidal pCO₂ levels of either normocapnic or hypercapnic conditions (Tafil-Klawe et al., 2004). Whereas these rodent models provide specific information on hypoxia or hypercapnia per se, they are, however, deficient in fully replicating real-life apneic conditions.

Since it is difficult to study many of the sleep apnea-induced neurophysiological and anatomical effects that often take decades to manifest, we used a method of inducing individual episodes of ventilatory arrest, i.e., apnea (mimicking the episodic desaturation, hypercapnia and tachycardia that accrue in patients with OSA (Beebe and Gozal, 2002), in in vivo guinea pigs, thereby allowing quantitative assessment of synaptic changes in the hippocampal CA3-CA1 pathway. In the present study, the animals were subjected to either single or recurrent episodes of apnea to 50% SpO₂ level that relates directly to nadir desaturation values reported in a population of obese OSA patients (Weiss et al., 2000) as well as those with severe lung disease (such as chronic obstructive pulmonary disease, COPD [Wynne et al., 1978]). Confirmatory morphological data, based upon single-stranded DNA immunolabeling, also demonstrate that clear signs of neuronal damage in CA1 occur, in guinea pigs subjected to recurrent apnea for a period of three hours. Other investigators have established, in rats, that a marked increase in apoptosis in CA1 is present after one to two days of chronic episodic hypoxia (Gozal et al., 2001). Of relevance to our hypothesis regarding glutamate excitotoxicity, it has been reported that apoptosis appears in retinal neurons at the 6th hour after a one-hour period of exposure to glutamate (Santos et al., 2001). More importantly, our immunolabeling results are consistent with the findings that DNA fragmentation is already present within three hours after oxygen-glucose deprivation in cultured CA1 neurons (de Pina-Benabou et al., 2005). These latter apnea-induced anatomical changes are consistent with findings from humans that indicate that there is a reduction in the thickness of the gray matters in the hippocampal CA1 region of OSA patients (Macey et al., 2002). Therefore, our in vivo guinea pig model of sleep apnea directly reflects the diagnostic signs and clinical sequelae of the OSA syndrome in humans. We hypothesize that the synaptic events that result in fEPSP potentiation that is accompanied by attenuation of the PPF (see below) following each episode of apnea in OSA may trigger glutamate-dependent receptor mechanisms, that when present chronically, are deleterious (excitotoxic) to CA1 neurons (Choi, 1988a,b; Godukhin et al., 2002). The latter concept is strengthened by our observation of apoptotic changes in CA1 neurons under the present in vivo experimental conditions of recurrent apnea.

3.2. Apnea-induced potentiation of the fEPSP is accompanied by attenuation of PPF ratios

In this study, we have detailed the temporal course of reversible changes in the apnea-induced potentiation of the CA1 fEPSP in the in vivo preparation. We demonstrated electrophysiologically that individual episodes of apnea produce a marked potentiation of the CA1 fEPSP within the first three minutes after the termination of apnea, with persistent potentiation lasting up to thirty minutes post-apnea. The increased fEPSP likely involves both presynaptic and postsynaptic mechanisms as both these neuronal elements are

subjected experimentally to the apneic insult. However, we believe a presynaptic effect of apnea plays a determinant role, at least in part, in the observed potentiation of the fEPSP based upon our paired-pulse analysis (see below).

A large body of literature suggests that PPF is mainly, but not exclusively (see (Wang and Kelly, 1996, 1997), of presynaptic nature (Harris and Cotman, 1985; Manabe et al., 1993). Specifically, classical hippocampal investigations have established that an increase in the presynaptic release of an excitatory (glutamate) transmitter is accompanied by a reduction in the paired-pulse facilitation (PPF) ratio of CA3-CA1 synapses (Andersen, 1960; Alger and Teyler, 1976; Creager et al., 1980; Hess et al., 1987). Patch clamp analyses also suggest that there is an inverse relationship between the amount of PPF and the initial transmitter release probability of the synapse (Manabe et al., 1993; Dobrunz and Stevens, 1997). Our present data indicate that the time courses of the fEPSP potentiation and the PPF ratio attenuation curves are similar, i.e., with overlapping peak effect and decay phases. Such correlative changes suggest that an increase in presynaptic release mechanisms underlie apnea-induced potentiation of the CA1 fEPSP. Hence, the paired-pulse analysis enables us to demonstrate, electrophysiologically, an increased synaptic efficacy in CA1, is consistent with an apnea-induced increase in glutamate release at CA3-CA1 synapses. In support of the hypothesis, *in vivo* intracerebral microdialysis studies suggested that the presynaptic release of glutamate at the CA3-CA1 pathway increases following cerebral ischemia (Benveniste et al., 1984; Mitani et al., 1994).

Quantitatively, the amount of maximal reduction in the PPF ratio (i.e., 25% and 16% for amplitudes and slopes, respectively, at two minutes post-apnea) is less than the amount of maximal potentiation of the fEPSP (i.e., 35% and 27% for amplitudes and slopes, respectively, at the same time point). This discrepancy suggests that postsynaptic factors also contribute to the apnea-induced increases in fEPSPs. Postsynaptically, there is evidence that an NMDA receptor-dependent hyperexcitability of CA1 neurons occurs after acute hypoxic exposure (Hori et al., 1991; Godukhin et al., 2002). Although various ionotropic and metabotropic receptors are known to be involved in the glutamate-induced excitotoxicity of central nervous system neurons (Choi, 1988a; Doble, 1999; Bonde et al., 2005), NMDA receptors are, in particular, one of the key determinants of apnea-induced potentiation of the CA1 fEPSP in the present study. We employed MK-801, a non-competitive, open-channel blocker of NMDA receptors, (Kemp et al., 1987; McAllister and Stevens, 2000) to identify the pharmacological nature underlying the apnea-induced changes in the synaptic responses of CA1 neurons. This drug was efficacious in blocking the apnea-induced potentiation of the fEPSP within one minute after ejection, which can be accounted for by its agonist-dependent properties (*ibid.*). MK-801 has also been reported to act with a similar rapid time course in blocking hippocampal NMDA receptors (Erdemli et al., 1990). The use-dependent behavior of MK-801 (Kemp et al., 1987; McAllister and Stevens, 2000) in antagonizing the NMDA receptor response of CA1 neurons to apnea suggests that glutamate agonists were present as a consequence of apnea. Since this drug selectively targets the abnormally high levels of NMDA receptor activation due to apnea, the blocking effect of MK-801 in Fig. 4 provides direct evidence in support of our hypothesis that there is an increased release of glutamate at hippocampal synapses after apnea which is also indicated, electrophysiologically, by an attenuated PPF, post-apnea.

3.3. Conclusion

The present data demonstrate that apnea promotes the presynaptic release of an excitatory transmitter (e.g., glutamate). We believe that the data from these studies establish a solid foundation for understanding the bases of various deficits in hippocampal functioning in OSA and breathing-related diseases and disorders of hypoxic origin. We hypothesized that chronic recurring episodes of apnea results in the excessive release of glutamate, and the

subsequent over-stimulation of postsynaptic glutamate receptors residing on CA1 neurons, likely contribute to structural (neurodegenerative) damages that underlie the functional (cognitive) deficits in patients with OSA. Thus, these data provide a window of opportunity to explore the degradative effects that arise vis-à-vis the electrophysiological processes and the morphology of neurons and synapses in the hippocampus using the in vivo guinea pig preparation. In addition, these data may provide avenues to develop effective clinical interventions with respect to pathologies that arise as a result of acute as well as chronic neurodegenerative processes including, but not limited to, periods of recurrent apnea during sleep.

4. Experimental Procedures

The Institutional Animal Care and Use Committee of the VA Greater Los Angeles Healthcare System (VAGLAHS) approved the experimental protocols. The guinea pigs used in these experiments were in good health as determined by veterinarians of the VAGLAHS. All experimental procedures were conducted in accord with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23, 1996).

4.1. Surgical preparation

Under isoflurane anesthesia, adult (3–9 months old) guinea pigs were tracheotomized. The jugular vein was cannulated for fluid and drug administration, and the animal was placed in a stereotaxic apparatus. Two holes (3 mm in diameter) were drilled in the skull, overlying the dorsal part of the hippocampus, to provide access for placement of electrodes to the stimulated CA3 and record from CA1 electrodes (Rapisarda and Bacchelli, 1977; Chirwa et al., 2001). After completion of all surgical procedures, alpha-chloralose (60 mg/kg, i.v.) was substituted for isoflurane. Supplementary doses of alpha-chloralose (30 mg/kg, i.v.) were administered as needed. During the experimental sessions, the guinea pigs were immobilized with gallamine triethiodide (0.2 mg/kg, i.v.) and artificially ventilated. Rectal temperature was maintained at 38–39°C by means of a heat lamp. Heart rate, and the hemoglobin O₂ level were continuously monitored by the pulse oximeter; respiratory end-tidal CO₂ was measured by a capnometer.

4.2. Recording, stimulation and data analysis

To record the monosynaptic, field excitatory postsynaptic potential (fEPSP), a glass microelectrode (2M sodium chloride-filled, 1–2 Megohms tip resistance) was positioned under stereotaxic guidance in the CA1 stratum radiatum that contains the dendritic field of pyramidal neurons (3 mm posterior, 2.5 mm lateral to bregma, 4 mm deep from cortex surface). A reference electrode consisted of a silver/silver chloride wire that was wrapped with saline-soaked gauze was placed subcutaneously. The signal from the recording electrode was amplified through an AxoClamp 2A preamplifier (Axon Instruments, Inc., Foster City, CA) operating in current clamp mode, and low-pass filtered at 2 kHz. The high-gain (100X) output signal was monitored on an oscilloscope and stored on a videocassette recorder equipped with a pulse code modulation (PCM) recording adapter (Vetter, Model 4000).

A stainless steel stimulating electrode was placed in the ipsilateral CA3 stratum radiatum (4.5 mm posterior, 4 mm lateral to bregma, 5 mm deep from cortex surface) in order to activate glutamatergic Schaffer collaterals and commissural fibers that synapse monosynaptically with CA1 pyramidal neurons (Johnston and Amaral, 1998). Single-pulse (0.2 ms duration) cathodal stimulation was applied with a Grass S88 stimulator (Grass Instruments, Quincy, MA) via a Grass photoelectric constant current isolation unit at 0.75–1 Hz, and electrode positions adjusted to maximize amplitudes of the fEPSP.

For each experiment, the optimal sites for CA3 stimulation and CA1 recording were determined by varying the locations of these electrodes independently. Once recording stability was achieved, the intensity-response (input-output) relationship of the CA3-CA1 synapse was determined by varying the stimulus intensities. Stimulation at an intensity eliciting approximately 50% of maximal fEPSP response was then applied continually (0.75–1 Hz) prior to and after single episodes of apnea. This determination ensured the accurate quantification of apnea-induced changes in the fEPSP in the absence of a ceiling effect or the inclusion of population spikes due to threshold discharges of CA1 neurons.

Apnea was induced by ventilatory arrest in order to obtain conditions of hypoxemia, hypercapnia, and tachycardia that correlate closely with those present in OSA patients (for details, see Discussion). Apnea was initiated that resulted in a desaturation of hemoglobin to 50% SpO₂; recovery to >95% SpO₂ occurred upon re-ventilation. Since the synaptic effects of an episode of apnea lasted up to 30 minutes (see Results), individual trials of apnea were separated by at least one hour throughout this study except that recurrent apnea protocols were adopted (instead of single-episodes of apnea) for the morphological studies (see below).

Double-pulse (0.2 ms, inter-pulse intervals 30–50 ms, repetition rate 0.75–1 Hz) cathodal stimulation of CA3's Schaffer collaterals/commissural fibers was used to elicit paired-pulse facilitation (PPF). Paired-pulse facilitation ratio was calculated as $[(fEPSP2 - fEPSP1) / fEPSP1]$, where fEPSP1 and fEPSP2 are the mean values of the first and second fEPSPs, respectively. The present study measured changes in both synaptic efficacy and the PPF ratio following single episodes of apnea; ventilatory arrest was performed to desaturate the hemoglobin to 50% SpO₂ levels while the fEPSPs were recorded.

The N-methyl-D-aspartate (NMDA) glutamate receptor antagonist MK-801 (100 μ M in saline, Sigma, St. Louis, MO) was used to determine the receptors that mediate the apnea-induced potentiation of the CA1 fEPSP. In order to locally administer the drug in the vicinity of the recording site in the stratum radiatum, a drug-ejecting micropipette (tip size: 10 microns) was affixed to the recording micropipette. Low-pressure microinjection (10 psi, 10 s duration) of the drug was performed one minute after the cessation of a single-episode apnea, which is the point in time wherein there is major potentiation of the fEPSP. The post-drug effect on CA1 fEPSP was compared to the pre-drug, potentiated condition that occurred as a result of apnea. MK-801 was also injected intravenously (2mg/Kg) to evaluate the effect of the global antagonism of NMDA receptors in the brain. The effect of the drug was evaluated by monitoring single-episode apnea-induced potentiation of the fEPSP 15 minutes post-drug in comparison to the pre-apneic control fEPSP.

Data were digitized off-line at 20 kHz (50 microseconds/bin) through a Digidata 1200 AD interface (Axon Instruments, Inc.) in combination with AxoGraph X Scientific Software. Both the amplitude and maximal slope of the fEPSP under control and apneic conditions were measured using the WaveStat functions of AxoGraph X Scientific Software. For determination of the amplitude and maximal slope of the fEPSP, the electrical potential preceding the onset of the stimulus artefact of either the first or the second stimulus of the CA3 was routinely taken as the baseline reference potential. The slopes of evoked fEPSPs were computed as the first derivative (i.e., volts per second) of fEPSP recordings. Data were analyzed statistically by two-tailed paired Student's *t* test (StatView 4.5 statistical software, SAS Institute, Cary, NC) to compare the control (pre-apneic) values with the experimental (post-apneic) variables. Differences were considered statistically significant at $p < 0.05$; data were expressed as means $\pm$ SEM.

4.3. Morphological study and immunohistochemistry

To determine the hypothesized excitotoxic effect of excessive synaptic release of glutamate associated with recurrent episodes of apnea, we evaluated morphological (apoptotic) changes in CA1 neurons in three animals that underwent three hours of recurrent apnea in comparison to a control group of animals with normal, normoxic, ventilation. Recurrent apnea consisted of the implementation of a cyclical sequence of apnea-ventilation-apnea that continued for three hours. Accordingly, apnea was induced by ventilatory arrest in order to produce a decrease in SpO₂ to the 50% level of oxygen saturation; recovery to >95% SpO₂ occurred prior to the initiation of each succeeding apneic episode. After three hours, the experimental and control animals were perfused transcardially with 100 ml of ice-cold saline (containing 200 units of heparin) followed by 300 ml of a fixative containing 4% paraformaldehyde, 15% saturated picric acid and 0.25% glutaraldehyde in 0.1 M phosphate buffer (PBS) (pH 7.4). The brains were removed and postfixed overnight in fresh fixative at 4°C. Tissue blocks of hippocampus were cryoprotected overnight in 20% sucrose (w/v) in 0.1 M PBS at 4°C; frozen sections of hippocampus were cut at 30–7µm thickness. For the assessment of apoptosis, sections were immunostained overnight with a mouse monoclonal antibody against single-strand DNA marker (Chemicon International, Inc., Temecula, CA; MAB3299; 1:10 dilution). This monoclonal antibody is a specific and sensitive marker of apoptosis (Frankfurt et al., 1996). Tissue sections were rinsed four times in PBS over 30 minutes prior to incubation for 90 minutes in PBS containing biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA; 1:300 dilution). Sections were then incubated for 90 minutes with the ABC complex (Vector Laboratories, Burlingame, CA; 1:200 dilution). They were then processed with diaminobenzidine (DAB) for 15–30 minutes in order to reveal the brown immunoreactivity of the single-stranded DNA in labeled (apoptotic) neurons. The sections were rinsed in PBS prior to mounting onto gelatin-coated glass slides; they were then dehydrated and coverslipped with Permount. At least one set of coronal sections from each animal was counterstained with Neutral Red before dehydration in order to identify hippocampal strata.

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Abbreviations

CA1	cornu ammonis region 1
CA3	cornu ammonis region 3
COPD	chronic obstructive pulmonary disease
fEPSP	field excitatory postsynaptic potential
NMDA	N-methyl-D-aspartate
OSA	Obstructive Sleep Apnea
PPF	paired-pulse facilitation
SpO₂	oxygen saturation level of hemoglobin

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Fig. 1.

Stimulus-evoked synaptic responses of hippocampal CA1 neurons as determined by the amplitude (A) and the maximal slope (B) of the fEPSP. Each data point in A and B is an average of 4 samples of the CA1 fEPSP evoked by stimulation of CA3 at graded intensities from 10 to 50 μ A. C: Superimposed averages of the CA1 fEPSP response to CA3 stimulus intensities at incremental steps of 10 μ A from 10 to 50 μ A. The traces from the top downwards are responses elicited by stimuli of the following intensities: 10, 20, 30, 40 and 50 μ A. Note that the latter two responses (evoked at 40 and 50 μ A) overlapped completely at their peaks. Stimulus artefacts (arrowhead) are truncated.

Fig. 2.

Time course of apnea-induced potentiation of CA1 synaptic responses as determined by the amplitude (A) and the maximal slope (B) of the fEPSP. Each data point represents the group mean value ($n = 12$ animals) of the fEPSP, recorded under control (CT, abscissa) conditions, and at post-apneic time intervals from one (1m, abscissa) to five minutes (5m, abscissa) after the termination of apnea (duration = 90 s, $SpO_2 = 50\%$). C–E: the averaged fEPSP ($n = 16$ traces) was potentiated at two minutes post-apnea (2.08 mV, 0.83 V/s) as shown in D compared with the pre-apneic control in C (1.54 mV, 0.65 V/s). E: recovery of the fEPSP occurred at 25 minutes after the termination of apnea (1.55 mV, 0.72 V/s). Stimulus artefacts (arrowhead) are truncated (intensity = 15 μ A). Symbols: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$. Number of trials = 24, 23, 17, 16, 13, and 7 for control, 1, 2, 3, 4, and 5 minutes post-apnea, respectively.

Fig. 3.

Time course of the apnea-induced reduction of the paired-pulse facilitation (PPF) ratio as determined by the amplitude (A) and the maximal slope (B) of the fEPSPs. Each data point represents the mean value ($n =$ eight animals) of the ratios of the PPF under control (CT, abscissa) and post-apneic conditions from one (1m, abscissa) to five minutes (5m, abscissa) after the termination of apnea. C and D: examples from one animal of averaged fEPSPs ($n =$ 16 traces) evoked by a paired-pulse paradigm (inter-pulse interval = 28.7 ms) under pre-apneic control (C) and post-apneic (D) conditions. Compared with the control (C), a marked reduction in the PPF ratios (D) occurred at three minutes after the termination of apnea (duration = 92 s, $\text{SpO}_2 = 51\%$). Stimulus artefacts preceding the synaptic responses are truncated (intensity = 35 μA for each pulse). Symbols: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$. Number of trials = 14, 12, 10, 12, 8, and 5 for control, 1, 2, 3, 4, and 5 minutes post-apnea, respectively.

Fig. 4.

Local ejection of MK-801 at the CA1 recording site blocks the apnea-induced potentiation of CA1 fEPSP responses. Following the development of the apnea-induced potentiation of the CA1 fEPSP (B), low-pressure microinjection (10 psi, 10 s duration) of MK-801 at the CA1 recording site totally blocked the apnea-induced synaptic changes within the first minute post-drug (C), resulting in a fEPSP of similar amplitude to that of the pre-apneic control (A). Each record from A to C is an average of 8 raw samples of CA1 fEPSP in response to stimulation of CA3 afferents (intensity = 30 μ A, duration = 0.2 ms). Stimulus artefacts are truncated (triangles).

Fig. 5.

Photomicrographs demonstrating positive labeling of single-stranded DNA immunoreactivity in hippocampal neurons of an experimental animal that underwent recurrent apnea (B), compared to the absence of single-stranded DNA immunoreactivity in the control animal (A). Positive neuronal immunolabeling (indicated by arrows) are mostly present in stratum pyramidale (p), with fewer labeling in adjacent strata oralis (o) and radiatum (r). Normarski optics. Calibration bars = 20 μ m.