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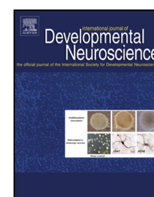

Cumulative effects of repetitive intermittent hypercapnic hypoxia on orexin in the developing piglet hypothalamus

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ABSTRACT

Orexin neuropeptides (OxA and OxB) and their receptors (OX1R and OX2R) are involved in maintenance of sleep and wakefulness, and are regulated by various environmental stimuli. We studied piglets, in the early neonatal period, exposed to 48-min of intermittent hypercapnic hypoxia (IHH; 7% O_2/8% CO_2) alternating with air. Three groups of 13–14 day-old piglets with IHH exposure of 1-day (1D-IHH) ($n = 7$), 2-days (2D-IHH) ($n = 7$) and 4-days (4D-IHH) ($n = 8$) were compared to controls (exposed only to air, $n = 8$). Immunoreactivity of OxA and OxB was studied in the piglet hypothalamic regions of the dorsomedial hypothalamus (DMH), perifornical area (PeF) and lateral hypothalamic area (LH). Results showed that after 1D- and 2D-IHH, total OxA and OxB expression decreased by 20% ($p \leq 0.005$) and 40% ($p < 0.001$), respectively. After 4D-IHH, the decrease in OxA and OxB was 50% ($p < 0.001$). These findings indicate that a chronic IHH exposure induces greater changes in orexin neuropeptide expression than an acute 1-day exposure in the hypothalamus. This may be causally related to the dysregulation of sleep.

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1. Introduction

The hypothalamic neuropeptides orexin-A and -B (OxA, OxB), also known as hypocretin-1 and -2, originate from cleavage of a putative precursor prepro-orexin (PPO) (De Lecea et al., 1998; Sakurai et al., 1998). They bind and activate two G-protein coupled receptors, orexin receptor-1 and -2 (OXR; OX1R and OX2R) (De Lecea et al., 1998), with OX1R having higher affinity for OxA and OX2R similar affinity for both OxA and OxB (Sakurai et al., 1998). Both OxA and OxB-immunoreactive (ir) neurons are concentrated in the lateral hypothalamic area (LH), dorsal medial hypothalamic (DMH) and perifornical area (PeF) regions of human, rat and pig hypothalamus (Sakurai et al., 1998; Thannickal et al., 2000; Ettrup et al., 2010), showing agreement with studies on the localization of PPO mRNA (Peyron et al., 1998; Sakurai et al., 1998; Nambu et al., 1999). Moreover, *in situ* hybridisation data indicates OXRs are widespread including the hypothalamus, thalamus, cerebral cortex and brainstem (Gotter et al., 2012; Alexandre et al., 2013).

Physiologically, orexin neurons are important for maintaining sleep, wakefulness and vigilance state in response to circadian, limbic and metabolic stimuli, via activation of monoaminergic and cholinergic neurons in the hypothalamus and brainstem (Sakurai, 2007). The extensive loss of orexin-synthesising neurons results in human narcolepsy (Thannickal et al., 2000), which is characterised by the symptoms of rapid onset of rapid eye movement (REM) sleep, impaired regulation of wake/sleeping state, excessive daytime sleepiness and possible abrupt loss of muscle tone (cataplexy) (Dauvilliers et al., 2007). Intracerebraventricular injection of orexin is also associated with arousal and feeding (Sakurai et al., 1998; Piper et al., 2000).

Orexin may also have a role in sleep regulation in two common sleep-related syndromes, obstructive sleep apnea (OSA) and sudden infant death syndrome (SIDS). Both have been shown to have decreased Orexin levels; decrease in serum in OSA patients (Igarashi et al., 2003; Sakurai et al., 2005) and decrease in the hypothalamus in SIDS (Hunt et al., 2015). Approximately 1–3% of children worldwide are affected by OSA (Lumeng and Chervin, 2008), whilst the latest SIDS rate in Australia (2012) was reported as 0.2/1000 live births (Statistics, 2014). OSA is characterised by

Abbreviations: DMH, dorsal medial hypothalamus; IHC, immunohistochemistry; IHH, intermittent hypercapnic hypoxia; ir, immunoreactive; LH, lateral hypothalamic area; OSA, obstructive sleep apnea; Ox, orexin; OxA, orexin A; OxB, orexin B; OXR, orexin receptor; OX1R, orexin receptor 1; OX2R, orexin receptor 2; PeF, perifornical area; SIDS, sudden infant death syndrome.

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repetitive, complete or partial episodes of upper airway collapse during sleep, leading to cessation of airflow resulting in intermittent hypoxia and hypercapnia (IHH) (Bradley and Floras, 2009; Chan et al., 2010). SIDS is the sudden unexpected death of a seemingly healthy infant under one year of age during sleep (Krous et al., 2004). Both OSA and two prominent risk factors of SIDS, prone sleeping and bed sharing, reproduce the re-breathing of expired air, thereby exposing the infants to repeated episodes of IHH (Thompson et al., 2006; Dwyer and Ponsonby, 2009; Harper and Kinney, 2010; Baddock et al., 2012).

Piglet models of IHH were developed in our laboratory in the 1990s, in order to determine the pathophysiology of IHH in pediatric OSA, and as a model of prone sleeping and bed sharing during infancy (Waters and Tinworth, 2001; Waters and Gozal, 2003). Piglets were studied at 10–13 days of age because at this age, physiological parameters of respiratory pattern behaviour and sleep/wake cycles (Scott et al., 1990), functional maturation and structure of the pulmonary vasculature (Hall and Haworth, 1986) and brain maturation rate (Dobbing and Sands, 1979) are equivalent to 2–4 month-old human infants, which is the peak incidence age of SIDS (Sullivan and Barlow, 2001; Aran et al., 2012). The physiological findings of the IHH model included an increase in the time to arouse and decrease in number of arousals, both of which were exacerbated after 4 days of IHH compared to 1 day of IHH (Waters and Tinworth, 2005).

In this study, we used only male piglets, given that SIDS (Mage and Donner, 2014), OSA (Lam et al., 2010) and reduced Ox neuronal numbers (Brownell and Conti, 2010) are all more prevalent in males. This study aimed to determine the effects of increasing duration of IHH on the immunohistochemical expression of OxA, OxB and OX1R and OX2R in the developing piglet hypothalamus and brainstem. However, we encountered problems with specificity of the OX1R and OX2R antibodies, and as such, we only present the data of the OxA and OxB in the hypothalamus. We hypothesised that OxA and OxB would decrease after 1 day IHH exposure and the decrease would be cumulative with subsequent days of IHH exposure thus being the cause of the greater reduction in arousability seen in the 4 day IHH piglets compared to 1 day IHH piglets.

2. Experimental procedures

2.1. Animal model

The live piglet experimental work, performed previously, has been detailed in Waters and Tinworth (2001) and Machaalani and Waters (2003). In short, mixed-breed miniature piglets were transported from a commercial piggery and were housed in a controlled 12:12 h light–dark cycle with access to food and water *ad libitum*. The experimental exposure was undertaken during the sleep (dark) period. Body weight was monitored daily. Ethical approval was given by the University of Sydney Animal Ethics Committee.

Piglets aged 10–13 days were exposed to either intermittent hypercapnic hypoxia (IHH) or air (control group). Piglets were harnessed, unsedated, into a vinyl hammock within a temperature-regulated perspex box. A full-face breathing mask was sealed against the snout, which was connected to a gastight three-way tap allowing rapid switching between reservoir bags containing air or the HH gas mix. The IHH exposure, 7% CO_2, 8% O_2 balanced with N_2 was delivered for 6 min, alternating with 6-min of air over 48 min (total 24 min of HH) per day. Delivery was given for 1, 2 or 4 days (1D-IHH, $n = 7$; 2D-IHH, $n = 7$; 4D-IHH, $n = 8$). The 1-day exposure was to mimic infants who were found to be prone sleeping on their stomach for the first time at the time of death (1st time prone sleepers), while the 2- and 4-day exposures mimic those who continually slept prone, bedshared, or suffered from OSA. The concentration of 7% CO2 and 8% O2 was used since this was found to induce similar physiological parameters (decreased arousals and decreased respiratory responses) and provide comparable blood gases, as seen in OSA infants (Waters and Tinworth, 2003, 2005). Moreover, this level of gas exchange was based on the findings of mechanical models of rebreathing where inspired CO_2 levels were found to range from 5 to 10% (Kemp and Thach 1993; Campbell et al., 1997). The control group ($n = 8$) received an air mix via the nasal mask, with tap switching but always in air, for either 1 ($n = 4$) or 4 ($n = 4$) days in the same study environment.

2.2. Tissue collection

Brain collection, tissue fixation and processing have also been described previously in Machaalani and Waters (2003). Euthanasia of the piglets was carried out with an overdose of pentobarbitone 24 h post final day of IHH treatment, and the whole brain was excised, down to the spino-medulla junction, weighed, and fixed in situ with 10% buffered formalin for 14 days. Fixing the brains in situ rather than via transcardial perfusion was performed to mimic the brain collection method of human autopsy material since our results are to be compared with those obtained from our SIDS tissue (Hunt et al., 2015). The brains were then sectioned into different brain regions at 4 mm intervals anterior to posterior, put into cassettes and returned into 10% buffered formalin for an extra 5 days. After washing in 70% ethanol (6–24 h), the cassettes were processed to paraffin in a Tissue Tek VIP 2000 processor (Ames Division, Miles Laboratories Inc., Indiana) by applying a 3-day automatic method, and then embedded in paraffin wax.

Tissue blocks at the level of the central tuberal hypothalamus, were sectioned at 7 μm by a rotary microtome (Shandon Finesse 325, Thermo Fisher Scientific Inc, Massachusetts, USA), and were mounted onto silanized microscope slides. Sections were dried overnight at 45 °C and stored at room temperature in a dust-free environment for a minimum of one week prior to immunohistochemical staining.

2.3. Immunohistochemistry

Immunohistochemistry was performed at room temperature. Tissue sections were first deparaffinised by 2 changes of xylene for 15 min each, followed by hydration in a graded series of ethanol: 100%, 100%, 95%, 70% to distilled H_2O. Heat-induced epitope retrieval was applied by microwaving on 'high' (Black and Decker, 700 W, USA) in 10% Tris-EDTA antigen retrieval buffer (1 mM EDTA, 1 mM sodium citrate, 2 mM Tris, pH 9.0) for 14 min. After cooling to room temperature and rinsing with distilled water and phosphate buffered saline (PBS; 0.01 M NaCL, 0.0027 M KCL, 0.14 M phosphate buffer; pH 7.4; Cat# 09-2051-100, Mediagoab, Uppsala, Sweden), a hydrophobic barrier surrounding the sections was drawn and endogenous peroxidase was quenched in Hydrogen peroxide (50% PBS, 50% methanol and 3% H_2O_2) for 25 min at room temperature, followed by two 3-min washes in PBS. Sections were blocked by 10% normal horse serum (NHS) in PBS for 30 min. Incubation with primary antibodies (Santa Cruz Biotechnology Inc., USA; Table 1) was left overnight at room temperature and dilutions used for OxA and OxB were 1:1000 in 1% NHS (made in PBS). Negative controls were covered with 1% NHS only. Details of these primary antibodies and their specificities are provided in Table 1.

Two 3-min PBS washes were undertaken prior to 45-min incubation with biotinylated secondary anti-goat antibody made in horse (Vector laboratories Inc., California, USA) prepared 1:2000 diluted in 1% NHS. Following two 3-min PBS washes, the sections were incubated with avidin-biotin complex (ABC) (VEPH4000, Vector Laboratories Inc.) for 30 min. The sections were then color-labelled with 3,3′-diaminobenzidine (DAB) (VESK3100, Vec-

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