Neuroscience 177 (2011) 35–42

SLOW INTRINSIC OSCILLATIONS IN THICK NEOCORTICAL SLICES OF HYPOXIA TOLERANT DEEP DIVING SEALS

J.-M. RAMIREZ,[a,b]* L. P. FOLKOW,[a] S. LUDVIGSEN,[a] P. N. RAMIREZ[a] AND A. S. BLIX[a]

[a]*Department of Arctic and Marine Biology, University of Tromsø, Breivika, NO-9037 Tromsø, Norway*

[b]*Department of Neurological Surgery, Center for Integrative Brain Research, University of Washington, 1900 9th Avenue, Seattle, WA 98101, USA*

Abstract—Direct evidence that the mammalian neocortex is an important generator of intrinsic activity comes from isolated neocortical slices that spontaneously generate multiple rhythms including those in the beta, delta and gamma range. These oscillations are also seen in intact animals where they interact with other areas including the hippocampus, thalamus and basal ganglia. Here we show that thick isolated neocortical slices from hooded seals intrinsically generate persistent spontaneous activities, both repetitive non-rhythmic activity with activity states lasting for several minutes, and oscillating activity with rhythms that are much slower (<0.1 Hz) than the rhythms previously described *in vitro*. These intrinsic activities were very robust and persisted for up to 1 h even in severely hypoxic conditions. We hypothesize that the remarkable hypoxia tolerance of the hooded seal nervous system made it possible to maintain functional integrity in slices thick enough to preserve intact neuronal networks capable of generating these slow oscillations. The observed activities in seal neocortical slices support the notion that mammalian cortical networks intrinsically generate multiple states of activity that include oscillatory activity all the way down to <0.1 Hz. This intrinsic neocortical excitability is an important contributor not only to sleep but also to the default awake state of the neocortex.

Key words: *Cystophora cristata*, neocortex, spontaneous neuronal activity, brain slices, hypoxia, diving.

The concept of the brain as the generator of intrinsic activity was first proposed more than a century ago (James, 1890; Brown, 1914). Today, intrinsic activity is widely considered as the driving principle of "simple" motor patterns in vertebrates and invertebrates (Lieske et al., 2000; Bucher et al., 2006; Marder and Goaillard, 2006) but its role in the generation of higher brain functions in mammals remains much less explored. Many studies of higher brain functions focused on the role of task or sensory evoked changes in brain activity (Raichle, 2010), creating the perception that many higher brain functions are primarily reflexive in nature. However, increasing evidence indicates that intrinsic activity is the major contributor to neocortical activity (Raichle, 2010; Binder et al., 1999; Roopun et al., 2008; Cunningham et al., 2006). In the intact brain neocortical activities interact with the hippocampus, thalamus and basal ganglia (Tort et al., 2008; Hahn et al., 2006; Wulff et al., 2009). Close to 95% of the brain's metabolic energy is devoted to the maintenance of intrinsic activities (Zhang and Raichle, 2010), and it is thought that complex tasks are shifting this intrinsic activity rather than activating neuronal network activity (Lakatos et al., 2008; He et al., 2009). The view of the brain as an intrinsic activity generator has dramatically changed our ability to characterize the neuronal substrate of complex behaviors and it opened the door to novel ways to explain and diagnose the neuronal basis of neurological disorders (Zhang and Raichle, 2010; Sorg et al., 2007; Greicius et al., 2007; Whitfield-Gabrieli et al., 2009; Monk et al., 2009). However, demonstrating the intrinsic nature of the brain's activity is always limited by the fact that the isolated brain cannot exist without constant oxygen and glucose supply. While neocortical, primarily neonatal, brain slices can be cut sufficiently thin to allow oxygen to penetrate the brain tissue via diffusion, these thin slices cannot preserve slow activity states that presumably require larger network interactions, such as the so called intrinsic default state (Raichle, 2010).

Using a medium that mimics the extracellular ionic composition *in situ* it is possible to readily obtain rhythmic recurrent activity (<1 Hz) in *in vitro* isolated neocortical slices, (Shu et al., 2003; Reig and Sanchez-Vives, 2007; Beggs and Plenz, 2003; Giugliano et al., 2004; Hoffman et al., 2007). A particularly robust intrinsic neocortical activity consists of self-sustained depolarized UP states, persisting for up to a couple of seconds and propagating to neighboring areas, followed by silent DOWN state periods (Shu et al., 2003; Steriade et al., 1993a,b; Timofeev et al., 2000; Sanchez-Vives and McCormick, 2000). Such self-maintained depolarized states may represent one of the basic operational states of local cortical networks that are important not only for regulating intrinsic neuronal excitability under various situations but also for sensory processing (Shu et al., 2003; Sanchez-Vives and McCormick, 2000; Castro-Alamancos, 2009; Haslinger et al., 2006; McCormick et al., 2003). Other operational states include rhythms in the alpha, beta and delta range (Roopun et al., 2008). However, none of the existing preparations generates an activity that resembles even closely that of the very slow oscillations in the infra-slow range. Such slow oscillations include the activity that gives rise to the BOLD signal and is thought to connect different regions of the

*Correspondence to: J.-M. Ramirez, Department of Neurological Surgery, Center for Integrative Brain Research, University of Washington, 1900 19th Avenue, Seattle, WA 98101, USA. Tel: +1-206-884-8188; fax: +1-206-884-1210.
E-mail address: Nino.ramirez@seattlechildrens.org (J.-M. Ramirez).
Abbreviation: aCSF, artificial cerebrospinal fluid.

0306-4522/11 $ - see front matter
doi:10.1016/j.neuroscience.2010.12.032

brain (Raichle, 2010; Monto et al., 2008; Hinterberger et al., 2003; Khader et al., 2008). Even in the intact brain, it is very difficult to electrophysiologically demonstrate these oscillations (Logothetis et al., 2009).

Here, we studied spontaneous neocortical neuronal activity in particularly thick (680–700 μm) hooded seal (*Cystophora cristata*) neocortical slices. Slices of this dimension are rarely used in studies of less hypoxia-tolerant adult mammals, due to the severe hypoxic conditions that prevail in their core, even during oxygenation (Bingmann and Kolde, 1982; Fujii, 1990; Fujii et al., 1982; Lipinski and Bingmann, 1986; Lipton and Whittingham, 1984). We found types of intrinsic spontaneous activity in the infra-slow range that have not previously been described in isolated brain tissue from mammals. Our finding suggests that these deep-diving mammals provide a unique opportunity to study neocortical network activity in the absence of the typical constrains set by the delicate oxygen sensitivity of the brain of terrestrial mammals. Seals are capable of diving to depths of ~1000 m for 1–2 h at a time (Folkow and Blix, 1999; Hindell et al., 1991). They tolerate drops of arterial oxygen tension well below 20 mm Hg (Elsner et al., 1970; Qvist et al., 1986; Meir et al., 2009). Their neuronal tissue is intrinsically very hypoxia tolerant (Folkow et al., 2008), which is in part explained by their remarkable specialization in oxidative metabolism (Mitz et al., 2009).

EXPERIMENTAL PROCEDURES

Animals

Experiments were performed using neocortical slices from 10 adult (sexually mature, 152±25 kg) female hooded seals (*Cystophora cristata*) that were culled for a range of scientific purposes in large hooded seal breeding colonies in the pack ice of the Greenland Sea, in conjunction with expeditions with the Norwegian research vessel "Jan Mayen" in March/April 2004 and 2005, under permits issued by Norwegian and Greenland authorities. The culling of 10 hooded seals out of a population of ~88,000 animals (Anonymous, 2006) is negligible and has no environmental impact. The seals were live-captured on the ice by use of hoop nets as previously described (Folkow and Blix, 1999), and subsequently decapitated in deep anesthesia (i.m. or i.v. injection of zolazepam/ tiletamine, 1.5–3.0 mg/kg of body mass (Zoletil Forte Vet, Virbac SA, France)), thus minimizing animal suffering. Use of a custom-made container laboratory allowed electrophysiological recordings to be performed on board the R/V "Jan Mayen" in immediate vicinity to the hooded seal colonies. The use of hooded seals was in accordance with the Norwegian Animal Welfare Act, as supervised by the National Animal Research Authority of Norway, and the number of animals used was reduced to a minimum.

Preparations

The brains were immediately isolated in ice-cold artificial cerebrospinal fluid (aCSF) bubbled with 95% O_2, 5% CO_2. The aCSF contained (in mM): 128 NaCl, 3 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 24 $NaHCO_3$, 0.5 NaH_2PO_4, and 30 D-glucose, pH of 7.4. The electrolyte composition of the aCSF matched that of hooded seal CSF, as determined in samples collected from five individuals (mean concentrations (in mM): 148 Na^+, 3.6 K^+, 125 Cl^-, 1.2 Ca^{2+}, 1.0 Mg^{2+} (Folkow et al., 2008)). Neocortical slabs were removed from the area of the visual cortex, glued onto an agar block and mounted in cold aCSF into a vibratome (OTS 3000, FHC Inc., Bowdoin, ME, USA). The slabs were oriented so that serially cut slices contained all neocortical layers as well as the underlying white matter (Fig. 1A). The hooded seal slices were 680 μm thick and typically 4–6 cm long, which covers an area multiple times larger than the sliced area of any previously generated neocortical slice. The entire dissection procedure up to the beginning of the slice production took less than 10 min. Slices were stored in aCSF at room temperature and continuously bubbled with 95% O_2, 5% CO_2. After an incubation time of approximately 60 min, individual slices were transferred into a submerged slice recording chamber where they were continuously superfused with oxygenated aCSF at a rate of ~30 ml/min and maintained at a temperature of 34 °C which is within the range of brain temperatures recorded in diving hooded seals (Odden et al., 1999; Blix et al., 2010). The temperature of the aCSF was maintained by means of a thermostatically controlled water bath that pumped water through a water jacket enclosing the recording chamber, and was continuously monitored using an isolated copper-constantan thermocouple connected to a digital thermometer (BAT 12, Physitemp, Clifton, NJ, USA). Severe hypoxia was introduced by bubbling the aCSF with 95% N_2, 5% CO_2 for 60 min, where after slices were re-oxygenated by bubbling the aCSF with 95% O_2, 5% CO_2.

The oxygen concentration in the aCSF of the recording chamber was measured with Clark-style oxygen tension (P_{O2})-sensitive electrodes (tip outer diameter 90 μm, Diamond General), connected to a polarographic amplifier (1900 Polarographic Amplifier, A-M Systems, Carlsborg, WA, USA). Before measurements, the electrodes were polarized for at least 1 h and calibrated in aCSF at 34 °C, saturated either with 95% O_2, 5% CO_2 or with 95% N_2, 5% CO_2. Tissue oxygen concentration measurements were also made near the slice surface and in its middle, by positioning the oxygen-sensitive electrode tip near or within the slice using a Leitz micromanipulator.

Recordings

Population activity and extracellular single-unit recordings were obtained from hooded seal brain slices with suction glass electrodes positioned on the surface of layer V of slices from the visual neocortex (Fig. 1A). The electrodes had a tip diameter of 50–80 μm for the population recordings and 10–40 μm for the single unit recordings. The extracellular signals were amplified 2000 times and filtered (low pass 1.5 kHz, high pass 250 Hz) using a Grass (P55, Grass Telefactor, West Warwick, RI, USA) and a Gould Universal (Gould Instrument Systems, Inc., Valley View, OH, USA) amplifier, and in case of population recordings the signals were rectified and integrated using an electronic filter (Paynter filter set at time constant of 30–50 ms). The electrodes were backfilled with the same aCSF that was used to superfuse the slices. Sites that exhibited spontaneous activity were located by moving the electrode to different sites along layer V. To avoid injury discharge we positioned the extracellular electrode very carefully on top of the slice surface using a micromanipulator (Leitz micromanipulator) that is commonly used for intracellular recordings. Before recording the extracellular activity at a particular site, we waited for at least 10 min to secure that the recorded activity was not induced by any injury discharge. Injury discharges were characterized by the sudden onset of high levels of action potential discharge associated with the positioning of the electrode. Injury discharges typically decayed over a period of 5 min, and such sites were always discarded. Each recording session lasted typically for >2 h of which 1 h was in severe hypoxia. We did not add any additional K^+ to the slices, nor did we decrease the extracellular concentrations of Ca^{2+} or Mg^{2+}.

In addition to the extracellular recordings we obtained also intracellular patch-clamp recordings from neocortical neurons of the pyramidal layer at variable depths (50–300 μm), using the blind-patch-clamp technique. For further details, see Folkow et al. (2008). Criteria for healthy neurons included proper action potentials, adequate input resistance and stable membrane potential for