Supplement 1. Respiratory neurons in culture

Respiratory neurons in culture demonstrated calcium spikes and waves (Supplementary Movie 3) which closely resembled those observed in the functional slice preparation. Notably, they were observed only in neurons which were isolated from the pre-Bötzinger complex (preBötC) but not from other regions of the 'rhythmic slice', from which the cells were isolated. For example, in six preparations we positioned the punching pipette in one half of the slice directly over preBötC and in another half its position was shifted 0.5 mm dorsal that included also *nucleus ambiguus*. The number of spontaneously active neurons in these two cell preparations were significantly different (75 ± 6 vs. 6 ± 3 per coverslip, n = 12). We prepared also the cultures of neurons from other brain stem nuclei, the nucleus of the solitary tract and hypoglossal nucleus (6 slices for each preparation used), and they showed no spontaneous activity.

Each coverslip contained 1,000–2,000 cells and we usually found three to five clusters of neurons which showed spontaneous activity. Inspiratory neurons are endowed by neurokinin (NK1), μ-opioid (mOR) and serotonin (HT-4a) receptors (Gray et al. 1999; Guyenet & Wang, 2001; Manzke et al. 2003; Pagliardini et al. 2005). Double-labelling experiments (NK1 vs. mOR and HT-4a vs. MAP2) were performed on 12 coverslips with cells from five animals. 43 % of cells (125 of 291) displayed intense NK-1 and mOR receptor co-immunoreactivity and 48 % of cells (136 of 282) revealed HT-4a receptor immunoreactivity that was comparable with cellular distribution of receptors obtained in slice preparation (Manzke et al. 2003).

Calcium transients were apparently related to the synaptic activity in culture. They were suppressed after blockade of synaptic activity with tetrodotoxin, ω-conotoxin, and after elevation of extracellular Mg^{2+} (Fig. 1S). Nifedipine also blocked oscillations, resembling its action *in vivo* (Mironov & Richter, 1998, 2000a). We examined the role of hyperpolarisation-

activated channels, which are implicated in generation of the rhythmic activity in some cell types (Harris-Warrick, 2002). Their selective blocker, ZD-7288 (30 μM), had no significant effect on calcium spikes that agrees with its action in the functional slice preparation (Mironov et al. 2000). Riluzole, a blocker of persistent Na^+ channels (Del Negro et al. 2002), did not inhibit the oscillations at concentrations up to 30 μM.

Ionotropic receptors for glutamate, glycine and GABA are important in the control of the respiratory rhythmogenesis. Actions of agonists and antagonists of these receptors *in vitro* (Fig. 2S) reproduced well the responses of the intact respiratory network (McCrimmon et al. 1986; Greer et al. 1993; Mironov & Langohr, 2005). Application of 1 μM AMPA transiently increased the amplitude and frequency of spikes after which they were suppressed. These effects correlated well with a rise in basal $[Ca^{2+}]_i$. Kainate (3 μM) and quisqualate (10 μM) acted similarly. NMDA (50 μM) increased the amplitude and the frequency of oscillations and its antagonist AP-5 (50 μM) decreased these variables. Calcium spikes were suppressed by the AMPA antagonist GYKI 53655 (50 μM), and AMPA/Kainate antagonists, CNQX and NBQX (both at 10 μM). The role of inhibitory transmission was examined by inhibiting $GABA_A$ receptors with 10 μM bicuculline and glycine receptors with 1 μM strychnine. Both antagonists transformed the rhythmic activity into irregular spikes mimicking the effects of inhibitors *in vivo*.

Respiratory rhythmogenesis is known to be modulated by various neurotransmitters acting as neuromodulators via G-protein-coupled metabotropic receptors, protein kinase A and protein kinase C (Bianchi et al. 1995; Johnson et al. 1996; Mironov et al. 1999; Mironov & Richter, 2000a,b). Both activation of adenylyl cyclase with 1 μM forskolin and inhibition of phosphodiesterase with 30 μM IBMX increased the frequency and the amplitude of $[Ca^{2+}]_i$ oscillations. After activation of $GABA_B$ and adenosine A_1 receptors with baclofen and CCPA (both applied at 1 μM), the transients were inhibited. Activation of metabotropic receptors

such as P_{2Y} receptors, metabotropic glutamate receptors, muscarinic receptors (10 μM carbachol), and NK-1 receptors induced Ca^{2+} mobilisation from ER. The effects of metabotropic receptors on calcium spikes were similar to those observed on the respiratory rhythm *in vivo*. During chemical hypoxia due to cyanide (Fig. 2Sj), the basal $[Ca^{2+}]_i$ increased and the spikes were abolished. The effects reproduced the actions of CCCP which induces Ca^{2+} efflux from mitochondria when oxidative phosphorylation is uncoupled (Mironov & Richter, 2001).

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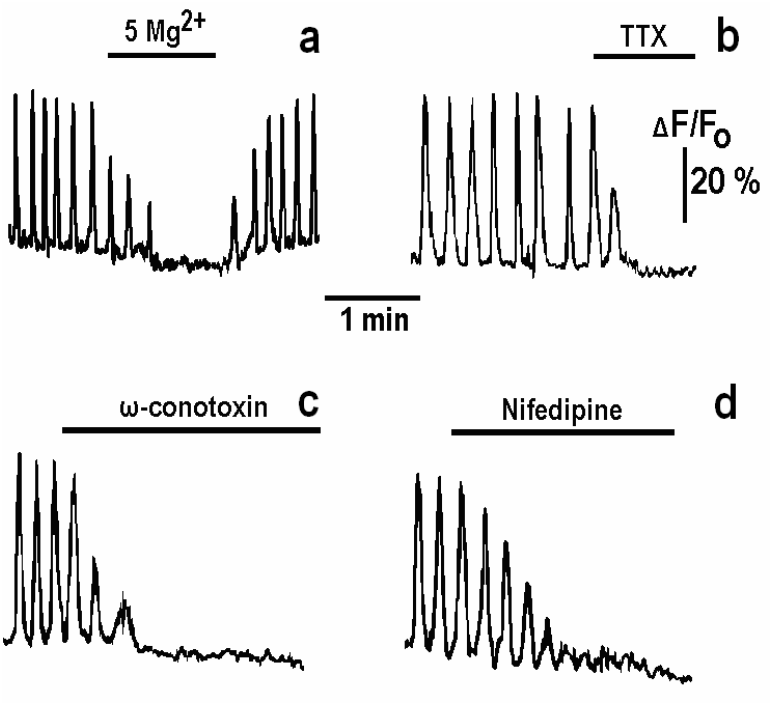

Fig. 1S. Synaptic activity and Ca^{2+} influx is required for generation of $[Ca^{2+}]_i$ spikes.

The effects of elevated (from 0.8 to 5 mM) extracellular $[Mg^{2+}]$ (**a**), the blockade of the fast Na^+-channels with tetrodotoxin (100 nM, TTX, **b**), N- and L-type Ca^{2+}-channels with ω-conotoxin (100 nM, **c**) and nifedipine (3 μM, **d**), respectively.

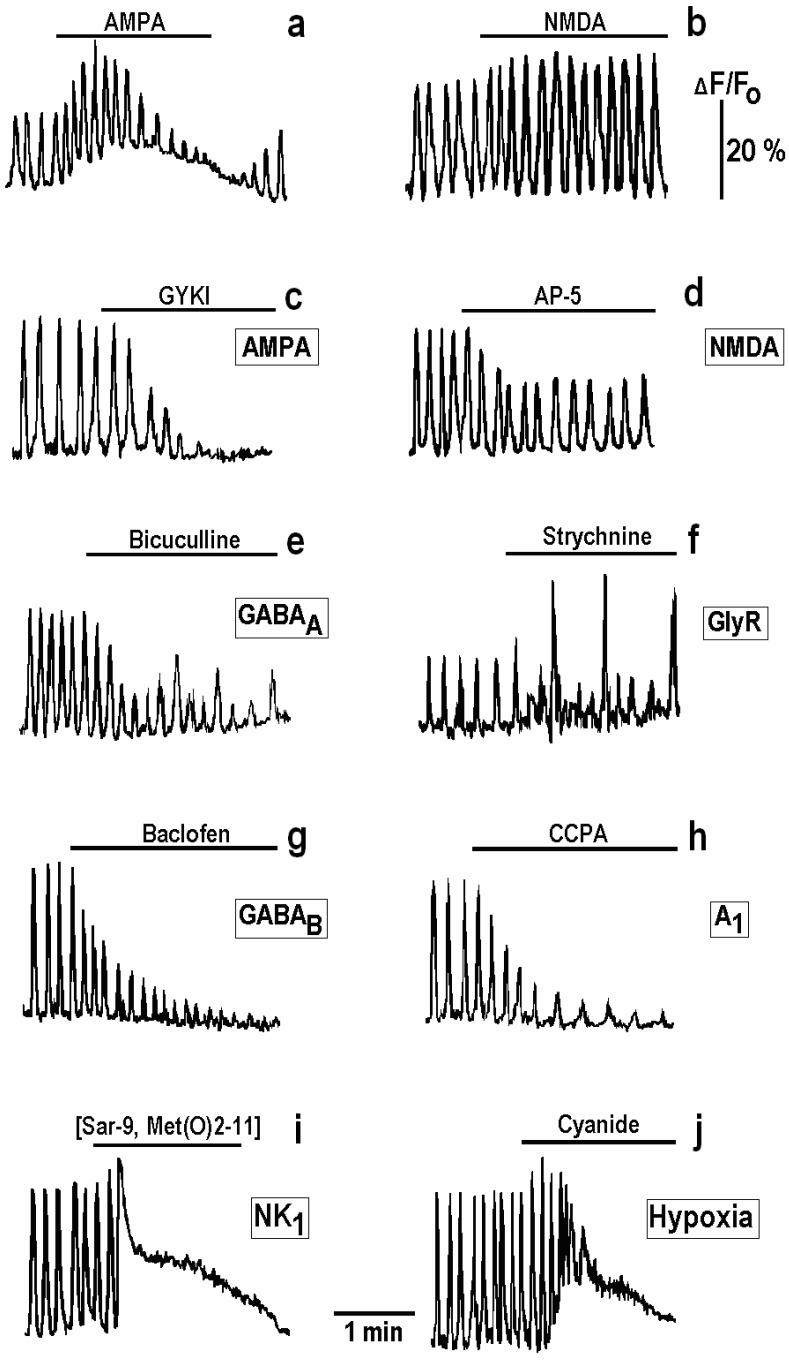

Fig. 2S. Modulation of $[Ca^{2+}]_i$ oscillations by ionotropic and metabotropic receptors.

Shown are the effects of agonists (**a**, **b**) and antagonists (**c**, **d**) of AMPA- and NMDA-types of glutamate receptors, respectively; the agonists of ionotropic $GABA_A$ (bicuculline, **e**) and glycine receptors (strychnine, **f**); metabotropic $GABA_B$ (baclofen, **g**), A_1 (CCPA, **h**), NK-1 ([Sar-9, Met(O)2-11], **i**) receptors; and chemical hypoxia induced by cyanide (**j**).