Supplement 2. Rhythmic activity depends on subthreshold depolarisation.

Rhythmic activity in brainstem slice preparation is commonly induced by moderate elevations of extracellular K^+ (Smith et al. 1991). In some slices (34 from 568 brainstem preparations), a stable respiratory motor output (RMO) was observed in standard ACSF at 3 mM $[K^+]_o$. During further increases in $[K^+]_o$, the activity was first augmented and then it was suppressed at 11 mM $[K^+]_o$ (Fig 3Sa). In the majority of slices, the rhythm was established only at $[K^+]_o >$ 6 mM, and RMO was suppressed at 13 mM $[K^+]_o$ (Fig 3Sb). In both types of the slices, the depression of the rhythm was accompanied by transformation of neuronal activity from bursting to the tonic firing (Fig 3Sb). We estimated, that the bursting appeared at $[Ca^{2+}]_i$ = 0.09 ± 0.02 μM and it was inhibited at $[Ca^{2+}]_i$ = 0.26 ± 0.03 μM (n = 12).

The dependencies of repetitive calcium spikes on membrane depolarisation were different in young and mature cultures (Fig 3Sd, e). At 3 - 6 DIV, the regular spikes were present in standard ACSF containing 3 mM $[K^+]_o$ and the rhythmic activity was suppressed at 11 mM $[K^+]_o$ (Fig 3Se). In mature cultures (15 - 18 DIV), the neurons showed no spontaneous activity and it was established only after elevation of $[K^+]_o$ to 6 mM. At 13 mM $[K^+]_o$, the transients were suppressed (Fig 3Se). Because spontaneous activity was observed more frequently in young cultures, this implicates its important role for the development, maintenance, and activity-dependent modulation of synaptic connections as observed in other types of neurons (Spitzer et al. 2002). Patch-clamp recordings showed, that increases in $[K^+]_o$ transformed the neuronal activity from quiescent through bursting to the tonically firing. Calibrating fluo-3 signal in terms of Ca^{2+} concentrations as described (Mironov & Hermann, 1994), we estimated, that the bursting appeared at $[Ca^{2+}]_i$ = 0.08 ± 0.02 μM and it was inhibited at $[Ca^{2+}]_i$ = 0.25 ± 0.03 μM (n = 12), similar to that observed *in vivo*. Such behaviour can also explain inhibition of persistent activity observed after elevation of extracellular Ca^{2+} concentration (Figs. 3Sc and f, respectively; see also Ruangkittisakul et al. (2007)) as well as

the responses obtained after activation of specific channels/receptors when the basal $[Ca^{2+}]_i$ was brought below or above this window of concentrations (Fig. 2S).

The existence of three modes of neuronal activity has been predicted by theoretical studies (Mironov, 1983; Butera et al. 1999; Kosmidis et al. 2004), showing that potential bursters possess a quiescent state, a tonically firing state, and the bursting mode. Transitions between the states proceed in a sequence (quiescent ↔ bursting ↔ tonic firing) and they can be induced by varying extracellular concentrations of K^+ or Ca^{2+}. Rhythmic activity correlated with changes in basal $[Ca^{2+}]_i$. Its appearance at $[Ca^{2+}]_i \sim 0.1$ μM is consistent with requirement of Ca^{2+} for activation of Ca^{2+} release channels. Neurons became tonically firing at $[Ca^{2+}]_i \sim 0.3$ μM, that possibly reflected the Ca^{2+}–dependent inactivation (desensitization) of Ca^{2+} release channels, (Mironov, 1994). Under normal conditions, inactivation helps to terminate the Ca^{2+} release by bringing the channels into the refractory state and sets the limiting frequency of $[Ca^{2+}]_i$ oscillations (Mironov & Usachev, 1991), but when the resting $[Ca^{2+}]_i$ is elevated, the CICR machinery is completely inactivated and the oscillations cease (see also a description of CICR model in Methods).

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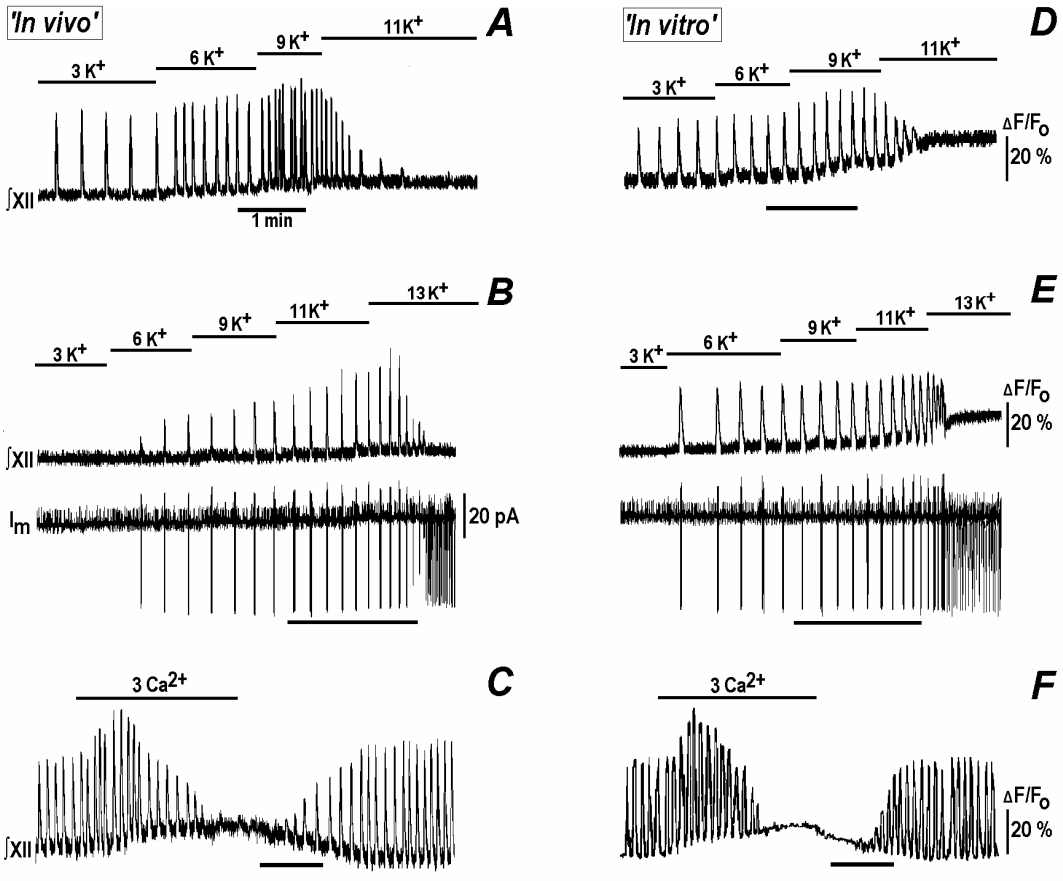

Fig. 3S. Modulation of rhythmic activity *in vivo* and *in vitro* by subthreshold membrane depolarisations and extracellular Ca^{2+}.

(***A***, ***B***) – Representative recordings in two preparations, in one of which the activity was present in the standard ACSF (***A***) and in another one the rhythm was established only after elevation of extracellular K^+ from 3 to 6 mM (***B***). (***C***) – Recordings were made at $[K^+]_o$ = 8 mM at $[Ca^{2+}]_o$ = 3 mM. (***D*** - ***F***) – Calcium spikes in the respiratory neurons at 3 - 5 DIV (**D**) and at 14 - 16 DIV (***E***) recorded during elevation of $[K^+]_o$ (***E***, and ***F***) and $[Ca^{2+}]_o$ (***F***). Note that progressive $[K^+]_o$ elevations increased the frequency and the amplitude of the inspiratory motor output (***A***, ***B***) as well as the calcium spikes (***D***, ***E***), until they were suppressed. (***B)*** and ***(E***) show also the cell-attached patch-clamp recordings of the membrane current (I_m). Note transformations of the inspiratory activity from bursting to the tonic firing during $[K^+]_o$ elevations. Time scale bar in all panels = 1 min.