D1

Electrophysiological recordings combined with Ca^{2+} measurements in subcellular compartments of specific neuronal phenotypes *in vitro*

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We have developed an *in vitro* model for studying electrophysiological properties and intracellular signalling in identified neuronal subpopulations, in particular GABAergic and noradrenergic (NAergic) neurones in the rat brainstem. To specifically visualise the neuronal phenotypes of interest, we use adenoviral vectors which express fluorescent proteins (such as EGFP) under control of cell-specific promoters. For targeting GABAergic neurones we use a 3.7kB part of the GAD67 promoter; NAergic neurones are targeted by the short synthetic PRSx8 promoter (see Teschemacher et al. 2005).

Sterile brainstem slices from humanely killed rat pups (p9-11) are transduced with viral vectors and cultured on Millipore organotypic membranes as previously described (Teschemacher et al. 2005). After 7-14 days, slice cultures are transferred into a glass bottomed recording chamber which is mounted on the stage of an upright laser-scanning confocal microscope equipped with DIC optics (Leica SP). The cultures are continuously superfused at 31±1°C with artificial cerebrospinal fluid.

EGFP-positive neurones are approached with patch pipettes filled with a solution containing the red-shifted Ca^{2+} indicator Rhod-2 (0.5 mM). Diffusion of EGFP into the recording pipette is visualised using the 488-nm wavelength line of an argon laser and confirms the successful establishment of whole-cell configuration with the targeted neurone. About 20 minutes after seal rupture, neurones are sufficiently loaded with Rhod-2 for measurements of intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) to commence. Rhod-2 is excited at 543 nm with a He-Ne laser and emitted light is sampled within the 560–640-nm band. Two-dimensional images can be scanned at 1-10 Hz. For higher temporal resolution, line scans focused on an area of particular interest can be employed at a frequency of about 2 kHz. For experiments with the green-emitting Ca^{2+} dyes, such as Oregon Green or Fluo 4, a viral vector expressing monomeric red fluorescent protein can be used.

We observe changes in $[Ca^{2+}]_i$ following electrical stimulation of the neurone via the recording pipette or by adding modulators of intracellular signalling cascades (such as nitric oxide donors or angiotensin II) to the bath solution. We analyse images off-line to extract information on differential $[Ca^{2+}]_i$ changes in subcellular compartments such as soma, dendrites, and putative axons and transmitter release sites. This technique enables us to directly study the details of intracellular signalling and electrical activity of defined neuronal phenotypes *in vitro* at high spatial and temporal resolution.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

D2

Use of an *in situ* artificially perfused whole rat preparation to explore central control of the sympathetic nervous system

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We have previously demonstrated the working-heart brainstem mouse preparation to the society (Paton 1996). This preparation has the upper thoracic spinal cord and associated sympathetic nerves. We have also demonstrated the trunk-hindquarters mouse preparation (Chizh et al. 1997)which has the caudal spinal cord and sympathetic outflow but lacks the brainstem, which is crucial for autonomic reflex integration. We have extended these *in situ* approaches to allow the whole rat to be artificially-perfused (Pickering et al. 2003). This preparation has intact brainstem-spinal cord-effector organ comunication and offers the advantages of access to caudal sympathetic outflows, the potential to monitor changes in organ system function (e.g. kidneys, bladder), a larger vascular tree and more options for peripheral sensory input (eg. hindlimb).

Wistar rats (50-150g) were deeply anaesthetised with halothane and the stomach, spleen and the bulk of the large and small bowel ligated and removed through a midline laparotomy. Access to the thoracic cavity was achieved via a midline sternotomy. After immersion in 4°C Ringers solution the animal was either decerebrated or decorticated (for studies of hypothalamic function). The animal was transferred to a recording chamber. A double lumen perfusion cannula was inserted via the left ventricle, through the aortic valve to sit in the first part of the ascending aorta. Carbogenated Ringers (32°C) containing either albumen (2%) or ficoll 70 (1.25%) was anterogradely perfused from a peristaltic pump at a rate of 20-30ml/min. Phrenic nerve activity (PNA) was recorded using a suction electrode. The preparation displayed rhythmic bursts of PNA and respiratory movements within 2 minutes of starting perfusion. The preparation was paralysed (vecuronium 2µg/ml) and the perfusate flow adjusted to obtain an optimal PNA pattern with ramping bursts signalling eupnoea and this was used to judge viability (up to 6 hours). The flow from the pump was computer controlled and can be flexibly adjusted to produce steps or ramps in perfusion pressure. Suction electrode recordings were made of the activity from the thoracic and lumbar chain, the renal, adrenal, mesenteric or splanchnic sympathetic nerves. Access to the brainstem and spinal cord is straightforward for either stimulation or recording.

The preparation shows Traub-Hering oscillations in perfusion pressure and also respiratory sinus arrhythmia indicating intact coupling between the brainstem respiratory network, the autonomic outflow and the end organs. All sympathetic outflows show pronounced respiratory modulation. Systemic perfusion pressure ramps demonstrated baroreflex sympathoinhibition. Stimulation of the chemoreflex (NaCN i.a.) evoked a striking increase in sympathetic activity and a corresponding increase in perfusion pressure. Application of a noxious mechanical or thermal stimulus to the hindlimb provokes increases in respiratory rate, perfusion pressure and sympathetic nerve activity.

This preparation is relatively quick to establish (30 minutes), permits straightforward access to the central and peripheral autonomic systems, allows excellent control over physiological conditions, has good stability for cellular recordings and provides an intermediate platform between in vitro and in vivo approaches for systems hypothesis testing.

Chizh B et al. (1997). Physiological Society, Bristol, UK 504P, 56P.

Paton J (1996). Physiological Society, Bristol 493P, 2P.

Pickering AE et al. (2003). J Physiol 551, 589-99.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.