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Temperature sensitivity of dopaminergic neurons in the Substantia Nigra pars compacta

K.K. Chung and J. Lipski, Department of Physiology, University of Auckland, Private Bag 92019, Auckland 1020, New Zealand.

Certain neurons in the CNS display a high temperature sensitivity (Q_{10}> 2.0) with respect to their firing frequency, e.g. in the hypothalamus, where such neurons are known to play a role in thermoregulation. There are reports of other brain regions expressing similar sensitivity, however, the cellular mechanism and pathophysiological significance of temperature sensitivity in extrahypothalamic neurons remain unclear. We hypothesise that this is due in part to the expression of temperature-sensitive ion channels in cell membranes. This hypothesis is supported by the recent discovery of a family of channels known as TRP (Transient Receptor Potential) channels (Minke & Cook, 2002). One member of the family (TRPV3), which is expressed both in the CNS and at the periphery, is sensitive to temperature changes in the physiological range (around 37°) (Xu *et al.*, 2002). In addition, this cation channel is relatively selective for calcium ions (Xu *et al.*, 2002), which suggests it plays a role not only in the control of neuronal excitability but also of intracellular Ca^{2+} homeostasis

The Substantia Nigra pars compacta (SNc) is a component of the basal ganglia important in motor control. Degeneration of this structure, associated with intracellular Ca^{2+} overload, leads to Parkinson's disease (Hirsch *et al.*, 1997). We have recently found that SNc neurons are temperature sensitive (Lipski *et al.*). The aim of the present study was to further characterise this sensitivity using a combination of whole-cell patch clamp recording and calcium imaging techniques and to explore what role, if any, TRP channels play in the temperature sensitivity of SNc neurons.

Transverse midbrain slices (250 µm) containing the SNc were obtained from young, anaesthetised Wistar rats and kept in aCSF bubbled with 95%O_2/5%CO_2. SNc neurons were visualised with IR-DIC (E600FN microscope, Nikon) and identified using a combination of morphological and electrophysiological criteria. Cells were patched with glass pipettes (2.5-5 MΩ) filled with a solution containing (in mM): 145 K-gluconate, 10 HEPES, 0.75 EGTA, 2 Mg_2ATP, 0.3 Na_3GTP, 2 $MgCl_2$, 0.1 $CaCl_2$, and held at −60 mV under voltage clamp. In some experiments, the Ca^{2+} indicator fura-2 (0.25 mM) was loaded into the cell by diffusion from the pipette solution. The level of free intracellular Ca^{2+} was monitored using the ratiometric technique (340/380 nm). Slices were maintained at 34°C except when temperature ramps were performed.

Transient cooling (by 2, 5 or 10°C) or heating (2 or 5°C) of the slice resulted in an outward (cooling) or inward (heating) current and corresponding changes in cell membrane resistance. The responses were fully reversible on return to control temperature (34°C). Temperature ramps with variable slopes demonstrated slow current kinetics. There was no sign of current desensitisation when steady-state temperature was reached. Cooling of slices by 5°C in the presence of ruthenium red (100 µM; a blocker of TRPV3 channel) produced a small reduction (22%; paired t-test $P<0.005$, n=5) of cooling-induced outward current. Ca^{2+} imaging experiments revealed temperature dependence of intracellular Ca^{2+} concentration consistent with the hypothesis that Ca^{2+} permeable channels are active at high temperatures and closed during cooling.

These experiments demonstrate a distinct pattern of electrophysiological and Ca^{2+} signal responses evoked by temperature changes in SNc neurons. Further studies are needed to confirm the involvement of TRP channels in temperature sensitivity and control of Ca^{2+} homeostasis in these neurons.

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The nature of non-linear interaction between P2 purinergic and α_1 adrenergic receptors in hypoglossal motoneurons is determined by the temporal pattern of receptor activation

R. Kanjhan[1,2], A. Jung[1], G.B. Miles[1], J. Lipski[1], G.D. Housley[1], M.C. Bellingham[2] and G.D. Funk[1],
[1]Department of Physiology, University of Auckland, Auckland, New Zealand and [2]School of Biomedical Sciences, University of Queensland, St. Lucia, QLD 4072, Australia.

Hypoglossal motoneurons (XII MNs) innervate the genioglossus muscle of the tongue and increase muscle tone during inspiration, thereby protecting airway patency. Their activity is constantly adjusted by a vast number of neuromodulatory systems to meet the changing demands placed on the respiratory system such as those accompanying changes in behaviour (suckling, vocalisation), environment (hypoxia) and arousal state (sleep-wake cycling). Reductions in XII MN activity and genioglossus muscle tone during sleep are believed to contribute to obstructive sleep apnea in adults and sudden infant death syndrome in newborns. Thus, there is considerable interest in understanding how modulatory systems alter the activity of XII MNs, particularly during sleep. Norepinephine (NE) potentiates XII MN activity primarily through activation of α_1 adrenergic receptors and subsequent blockade of a resting K^+ conductance. Reduced release of NE during sleep is believed to contribute to a reduction of XII MN activity, airway instability and apnea. Extracellular adenosine 5'-triphosphate (ATP) also potentiates inspiratory XII motor output through activation of P2 receptors. A likely source of ATP is via co-release with NE. The goal of this study was to explore how NE and ATP signaling systems interact to affect XII MN activity and inspiratory motor output. Phenylephrine (PE, α_1 adrenergic receptor agonist, 1-100 µM) and ATP (0.1-10 mM) were applied alone and together (in the presence of 100 µM theophylline), either simultaneously or sequentially, to the XII nucleus of medullary slice preparations that continue to generate a respiratory-related rhythm *in vitro* following isolation from anaesthetised neonatal rats.

Bath or local application (15-30s) of PE or ATP alone potentiated ipsilateral XII inspiratory nerve output (n=6), and activated inward currents in whole-cell voltage-clamped XII MNs (n=90). PE responses developed slowly and lasted for many minutes. ATP responses comprised a rapid-onset excitatory component, presumably mediated by P2X receptors. These fast, ATP-gated inward currents were further classifi ed according to their desensitisation kinetics as fast-, slow- and non-desensitising. The excitatory phase was followed in some cases by a slow onset, inhibitory component which manifest as a decrease in burst amplitude or a small amplitude outward current, suggestive of a P2Y receptor mechanism. To assess interactions, we compared the magnitude of currents induced by PE and ATP alone and in combination. ATP, when applied prior to PE, attenuated the PE current to 62% of control (n=32; $p<0.01$), suggesting a negative interaction. Surprisingly, when PE was applied prior to ATP, a positive interaction was observed. In 45% of MNs (16 of 35), particularly those showing non-desensitising responses to ATP, PE caused up to a 3-fold potentiation of both the fast-inward and slow-outward components of the ATP current. That this did not simply reflect differences between MNs was demonstrated in 5 MNs (in 1 µM TTX) where both positive and negative interactions were produced by switching the order of agonist application.

In summary, we have defi ned a novel mechanism where the nature of the non-linear interaction (positive vs. negative) between two neuromodulatory signaling cascades is determined by their temporal pattern of activation. While the underlying pathways remain to be determined, it is clear that such a mechanism will dramatically increase the dynamic range over which a given modulatory input can modify neuronal excitability.

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Glutamate alters the morphology of dendritic spines in motoneurons

A.J.C. McMorland[1], D.M. Robinson[1], C. Soeller[1], M.B. Cannell[1] and G.D. Funk[2], [1]Physiology, University of Auckland, New Zealand, and [2]Physiology, University of Alberta, Canada.

Dendrites are the primary receptive component of neurons. In motoneurons (MNs), for example, the dendritic tree comprises ~90% of the membrane surface area (Cullheim *et al*., 1987), up to 60% of which is covered with presynaptic terminals (Örnung *et al*., 1998). Dendrites therefore play a primary role in determining neuronal information processing. The first opportunity for dendritic signal processing is at the synapse. In a variety of neuron types, synapses occur on spines, specialised membrane projections where the effect of a synaptic input on the cell can be dynamically regulated by altering spine morphology. Spines have only rarely been reported on MNs. As an initial step toward understanding the functional significance of spines for MN function, we combined laser-scanning fluorescence microscopy, whole-cell recording and image processing techniques to characterise the density, distribution and dynamic morphology of dendritic spines in hypoglossal (XII) MNs from neonatal mice.

Medullary slice preparations (200 µm thick) were prepared from anaesthetised Swiss CD-1 mice (postnatal day 0-3) and XII MNs were labelled during whole-cell recording using pipettes filled with avidin-conjugated biocytin (Molecular Probes). For morphological experiments, tissue was fixed (4% paraformaldehyde), and recorded cells were visualised using Alexa 488-avidin conjugate (Molecular Probes) and imaged using confocal microscopy.

Dendritic segments displayed two distinct morphological patterns: (Type I) smooth, uniformly tapered segments with spines at low density (0.09 ± 0.01 spine.µm^{-1}, n = 3), or (Type II) periodically swollen segments that lacked spines. Spines also displayed a range of morphologies, including the classical mushroom-headed (pedunculated) form (site of synaptic inputs in other neurons) and those characterised by long filopodia but no head (proposed to be important for synaptogenesis; Cailliau Portera & Yuste, 2001).

We then tested the hypothesis that spine structure is not fixed but can be dynamically modulated in response to glutamate receptor activation. Moreover, based on observations in cultured cortical neurons (Hasbani *et al*., 2001), we hypothesised that the Type II morphology results from retraction of spines and local swelling of dendrites at these sites in response to high concentrations of glutamate. XII MNs were labelled with Alexa Fluor 350 or 488 Hydrazide (Molecular Probes) under voltage-clamp conditions. Spines were identified and their morphologies recorded before, during and after bath-application of glutamate (125 - 500 µM for 5 - 10 minutes), using two-photon excitation microscopy. Glutamate application was associated with membrane depolarisation (from -59 ± 7 mV, to -5 ± 0.7 mV, n = 2), spine retraction and dendritic swelling, which transformed dendrites from Type I to Type II as defined in fixed preparations. These changes partially reversed upon glutamate wash-out. Membrane potential returned to -52 ± 2 mV and dendritic swelling was reduced.

These data confirm the presence at low density of dendritic spines on XII MNs, and establish that their morphology is not fixed but can be modified by glutamate receptor activation. Whether these changes in spine morphology represent a physiological mechanism important for synaptogenesis, for modulating synaptic strength (Hasbani *et al*., 2001) or reflect the pathological consequence of glutamate-induced excitotoxicity remains to be determined.

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μ and δ opioid receptor mRNA and protein expression in the cerebellum of the foetal, neonatal, and adult rat

J.H. Miller, E.M. Mrkusich, B.M. Kivell and D.J. Day, School of Biological Sciences, Victoria University of Wellington, P.O. Box 600, Wellington, New Zealand.

The three classical opioid receptors, μ, δ, and κ, are found in many regions of the rat brain where they act to modulate neurotransmission in the adult and regulate neurogenesis in the fetus and neonate. Based on radioligand binding and autoradiography, it is generally accepted that within the lobes of the rat cerebellum only δ opioid receptors are expressed. This is in contrast to the situation in humans and rabbits in which both μ and δ receptors are expressed in the cerebellum. Using frozen, paraformaldehyde-fixed cerebellar sections from foetal, neonatal, and adult Wistar rats, we investigated μ and δ opioid receptor protein distributions by immunohistochemistry, and opioid receptor mRNAs by fluorescent *in situ* hybridisation. Immunohistochemical staining using commercially available μ and δ antibodies followed standard procedures. For *in situ* hybridisation, riboprobes directly labeled with a fluorescent marker were used, thus, allowing comparative quantification of the message in brain tissue sections. cRNA probes complimentary to the 5' untranslated region of the mRNA were prepared. Targeting of this region rather than the coding region minimised cross hybridisation of cRNA probe between the closely related opioid receptor mRNAs that share significant regions of sequence similarity in their coding regions. Labeled cRNA probes were prepared from T7-tailed PCR products by *in vitro* transcription with T7 RNA polymerase and incorporation of Cy3-UTP or fluoroscein-UTP into the reaction mixture. The results of this study showed that both μ and δ opioid receptor proteins and mRNAs were present in the adult and six day old neonatal rat cerebellum, specifically within Purkinje cells and in the granular layer. Expression of μ opioid receptor mRNA was also detected within cells of the molecular layer, but at lower levels than those seen within the Purkinje cells. Abundant expression of μ and δ opioid receptor mRNAs was also detected in the external germinal layer of the immature cerebellum of the foetal sixteen day post-conception rat, a finding that suggests a role for opioid receptors in neurogenesis of the developing cerebellum. Identification of both μ and δ opioid receptors within the developing cerebellum, and the known role of the cerebellum in coordinating multi-joint movements, supports the hypothesis that opioid receptors and their ligands affect development of coordinated movements in the neonate.

Upregulation of ecto-nucleoside triphosphate diphosphohydrolases 1 and 2 in noise-exposed rat cochlea

S.M. Vlajkovic[1], G.D. Housley[1], D.J.B. Munoz[2], S.C. Robson[3], J. Sevigny[4], C.J.H. Wang[1] and P.R. Thorne[2], [1]Department of Physiology, Faculty of Medical and Health Sciences, The University of Auckland, Auckland, New Zealand, [2]Discipline of Audiology, Faculty of Medical and Health Sciences, The University of Auckland, Auckland, New Zealand, [3]Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA and [4]Centre de Recherche en Rhumatologie et Immunologie, CHUQ, Université Laval, Sainte-Foy, Québec, Canada.

Extracellular ATP acting via P2 receptors in the inner ear initiates a variety of signalling pathways that may be involved in noise-induced cochlear injury (Thorne *et al*., 2002). NTPDase1/CD39 and NTPDase2/CD39L1 are key elements for regulation of extracellular nucleotide concentrations and P2 receptor signalling in the cochlea (Vlajkovic *et al*., 1999, 2002). This study characterised the effect of noise exposure on regulation of NTPDase1 and NTPDase2 expression in the cochlea using a combination of real-time RT-PCR, immunohistochemistry and functional studies. Adult Wistar rats were exposed to broad band noise at 90 dB and 110 dB sound pressure level (SPL) for 72 hours. Their auditory function was assessed by auditory brainstem response to clicks and pure tones. Exposure to 90 dB SPL induced a small and temporary change of auditory thresholds (temporary threshold shift), whilst exposure to 110 dB SPL induced a robust and permanent change of auditory thresholds (permanent threshold shift). NTPDase1 and NTPDase2 mRNA transcripts were upregulated in the cochlea exposed to 110 dB SPL, whilst mild noise (90 dB SPL) altered only NTPDase1 mRNA expression levels. Changes in NTPDase expression did not correlate with levels of circulating corticosterone, implying that the upregulation of NTPDase expression was not stress-related. Quantitative immunohistochemistry in the cochlea exposed to 110 dB SPL localised the increased NTPDase1 and NTPDase2 expression in the stria vascularis and upregulation of NTPDase2 in the intraganglionic spiral bundle. Whilst NTPDase1 was upregulated in the secretory tissues of the lateral wall, it was down-regulated in the cell bodies of the spiral ganglion neurones. Tissue distribution of NTPDases was not altered in the cochlea exposed to 90 dB SPL, implying a differential regulation of NTPDase expression in the cochlea in response to different noise levels. Functional studies revealed increased ectonucleotidase activity in the cochlea after exposure to 110 dB SPL, consistent with upregulation of NTPDases. These data indicate that the regulation of NTPDase1 and NTPDase2 expression in the cochlea is responsive to noise as a stimulus that also upregulates P2 receptor signalling pathways. The changes in NTPDase expression may reflect compensatory responses of cochlear tissues to limit ATP signalling during noise exposure and protect the cochlea from noise.

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Quantifying local diffusion in the rat lens by two-photon flash photolysis

M.D. Jacobs, C. Soeller, M.B. Cannell and P.J. Donaldson, Department of Physiology, The University of Auckland, Private Bag 92019, Auckland, New Zealand 92019.

The vertebrate ocular lens is an avascular organ composed of fibre-shaped cells that run from the anterior pole to the posterior pole of the lentoid mass. Often measuring several millimetres or more in diameter, the lens cannot rely on passive diffusion alone in order to maintain homeostasis and transparency. A model of lens micro-circulation has been proposed based upon external electrophysiological, and biochemical data (Donaldson *et al.*, 2001). However, few functional studies to date have focused on transport mechanisms at varying depths within the lens mass. As a transparent tissue composed of highly ordered and regularly-shaped cells, the lens is an ideal system for studying intra- and intercellular transport by optical methods. We have applied two-photon microscopy and image analysis to lenses loaded with a caged fluorescent dye, in order to quantify local diffusion within and between fibre cells at varying depths. We have compared these functional results with our previous structural studies (Jacobs *et al.*, 2001) to elucidate structure-function relationships of fibre cell transport.

Lenses were extracted from adult rats killed by CO_2 asphyxiation in accordance with protocols approved by The University of Auckland Animal Ethics Committee. Lenses were cut in half through the equator and placed in a perfusion chamber containing intracellular medium and 1mM fluorescein bis-(5-carboxymethoxy-2-nitrobenzyl) ether (CMNB-caged fluorescein). The chamber was mounted on the stage of a confocal microscope modified for two-photon excitation. The two-photon laser beam bypassed the scanning system of the microscope and was focused inside a selected fibre cell to uncage the fluorescein by two-photon flash photolysis. Movement of the fluorescein away from this point source, both within and between cells, was imaged in real-time using confocal optics in x-y and line-scan modes. Data were written to hard disk and quantitative analysis of dye movement was performed using custom-written software.

In the lens periphery the spread of the uncaged fluorescein was highly directional, corresponding to radial rows of fibre cells. Deeper in the lens (>300 µm) the cell-cell coupling was approximately isotropic around the cell targeted for photorelease. The directional cell-cell coupling observed at the periphery corresponded to the local expression pattern of gap junctions on opposite broad sides of the hexagonal fibre cells. The isotropic coupling deeper in the lens corresponded to the dispersal of gap junctions in older fibre cells. Quantitative image analysis allowed characterisation of the time courses of differential dye transfer between neighboring fibre cells in different regions of the lens. Cytosolic fluorescein diffusion coefficients were estimated by fitting diffusion equations and by comparison with numerical simulations. Taken together, our functional and structural data are consistent with a lens micro-circulation model in which gap junctions near the lens equator facilitate solute transport to the lens surface where appropriate ion channels and transporters are concentrated.

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