452P MECHANISM OF NERVE CELL DEATH IN PARKINSON'S DISEASE

E. Hirsch, INSERM U289, Hôpital de la Salpêtriére, Paris, France.

Parkinson's disease is characterised by a loss of dopaminergic neurons in the substantia nigra. However, not all dopaminergic neurons generate the same extend in this disease. Among several other factors, the neurons which are vulnerable to Parkinson's disease have been shown already in the control mesencephalon to produce a high amount of oxygen free radicals and to be poorly protected against oxidative stress. However, the cause of neuronal loss in the substantia nigra in Parkinson's disease remains unknown. It has been hypothesised that deleterious free radical production might play a role in the death of dopaminergic neurons which ultimate dye by apoptosis. Glial cells may also participate in the mechanisms of nerve cell death by producing cytokines such as tumor necrosis fact α. Indeed, microglial cells producing TNF α are observed in the substantia nigra of patients with Parkinson's disease. Furthermore, dopaminergic neurons in the human substantia nigra express tumor necrosis factor α. In vitro experience on primary mesencephalic cultures of rat embryos show that the activation of this pathway produces apoptosis via the synthesis of oxygen free radicals and the translocation of NF-Kb transcription factor. Post mortem data observed in the parkinsonian mesencephalon also suggests that NF Kb translocation to the nucleus, an index of it activation is observed under the pathological circumstance. These data suggest that this oxidant-mediated apoptogenic transduction pathway may play a role in the mechanism of neuronal death in Parkinson's disease.

453P FROM THE FROG ROOM TO THE RAT MOTO-NEURONE: RECEPTOR MECHANISMS IN CULTURED MOTONEURONES

D I Wallis, P Elliott, G A Foster & B M J Stringer*, Physiology Unit, UWCC, Cardiff CF1 3US and *Dept. of Oral Pathology, Sheffield University, Sheffield S10 2TA

In the spinal motoneurone (MN) considerable interaction of neurotransmitters and integration of convergent signals occur. Culturing embryonic MNs with reduced dendritic trees allows easier access to patch electrodes, quantitative pharmacology and a readier investigation of receptor mechanisms. Trophic factors appear to allow survival and maturation of various features present in adult MNs, but these factors are mainly unidentified.

Our long-term cultures of ventral horn neurones from embryonic 14 day (d) rats were enriched by density gradient centrifugation to give >85% cells with ciliary neurotrophic factor (CNTF) MN characteristics. They were grown with ciliary neurotrophic factor (CNTF) on spinal cord glial monolayers for up to 90 d and investigated using patch clamp. The expected profile of MN features includes expression of choline acetyltransferase (ChAT) and calcitonin gene-related peptide (CGRP), voltage-activated currents such as I_A, I_h and I_{Ca}, neurotransmitter-generated currents e.g. to EAAs and 5-HT; possibly also firing properties reflecting neural connections.

Over 85% of neurones were ChAT-positive and a network of CGRP-positive processes was seen. Several types of K current were displayed, including a sustained, outward current (IDR, peak current 6.5±2.4 nA, peak current density 92.1±36.6 pA/pF, means and s.e. means, n=28) and a transient I_A current (peak 4.0±1.2 nA, peak current density 64± 12 pA/pF, n=7). The mixed cation current, I_h, was present in all but the earliest cultures and increased with time in culture. Ih was blocked by 2mM Cs^+ and showed voltage-dependent activation ($V_{0.5}$ -95 ± 8mV, slope-8.6±1.4, n=4). In 12 mM K^+ medium, 5-HT (1-10 μM) potentiated I_h current in 6 cells investigated. Tryptaminergic projections have not developed by d 14 of gestation. It was uncertain whether 5-HT receptors would be expressed. Depolarizations to 1-10 μM 5-HT were initially small or absent, but increased with time in culture up to amaximum of 18mV after 70 d. They were blocked by 0.1 μM ketanserin. 5-HT (1-10 μM) evoked an inward current whose magnitude was positively correlated with culture time ($r = 0.39$, $P<0.01$, mean 147.8±15.3pA, n=48). Most 5-HT-induced current seemed to result from block of a leak conductance and enhancement of a Na^+-sensitive conductance other than I_h; enhancement of I_h may contribute in some cells. Ba^{2+} (2mM) occluded 5HT responses and itself induced an inward current unaltered by time in culture. Synaptic activity with an irregular bursting pattern occurred. Addition of strychnine (10 μM) or bicuculline (30 μM) induced rhythmic patterned bursts, reminiscent of recordings from *in vitro* rat spinal cord and probably triggered by disinhibition.

Coupling mechanism and the expression of peptide receptors have yet to be explored. Staged removal of trophic support shows that MNs will survive without CNTF in medium conditioned by glial cells, contact with which is not necessary. Which features depend for their expression upon synaptic input and/or Ca^{2+} entry have still to be explored.

Frances M. Leslie University of California, Irvine, USA

Nicotine, a psychoactive alkaloid derived from tobacco, interacts with nicotinic acetylcholine receptors (nAChRs) to produce its biological effects. Recent cloning data indicate that neuronal nAChRs consist of combinations of α (α2-α9) and β (β2-,β4) subunits. Prenatal exposure to either cigarette smoke or nicotine is associated with significant alterations in brain development. However, with the complexity of *in vivo* systems, it is difficult to assess underlying mechanisms. In the present study, we have used *in vitro* techniques to determine whether nicotine has direct effects on developing brain. We have examined the localization of nAChRs in developing rat brain, using radioligand binding and *in situ* hybridization. Our data indicate that nAChRs are expressed in brain early in gestation. In many brain areas, particularly sensory regions, there is a transient upregulation of nAChR expression which coincides with the major phase of synaptogenesis. α7 nAChRs, which have been shown previously to be very permeable to calcium, are highly regulated by a number of developmental factors. Removal of excitatory thalamic afferents rapidly decreases α7 nAChR mRNA and protein levels within the somatosensory "barrel" cortex, indicating a tight transsynaptic regulation. In addition, two mechanisms have been identified by which glia inhibit α7 nAChR expression: (1) release of a glia-derived factor and *(2)* removal of glutamate, which has a stimulant effect on nAChR binding.

Using cultured locus coeruleus (LC) neurons as a model system, we have examined the functional status of nAChRs in fetal rat brain. Cells derived from gestational day *14* rostral rhombencephalon, were cultured for four days *in vitro,* then incubated with [^{3}H]norepinephrine (NE). Nicotine stimulated [^{3}H]NE release from cultured LC neurons in a calcium-dependent manner. Pharmacological analysis that this effect was mediated by a nAChR which consisted of α4 and 2 subunits. Whereas the stimulant effects of nicotine and NMDA on [^{3}H]NE release were not additive, the two agonist responses exhibited distinct pharmacological profiles. Thus, nicotine stimulated [^{3}H]NE release was not mediated indirectly via stimulation of neuronal glutamate release. Whereas previous studies have suggested that nicotine effects on brain catecholamine development are indirect, our present findings indicate that nAChRs directly regulate NE release in fetal brain. These findings are consistent with the hypothesis that nicotine acts directly on fetal central nervous system to modify subsequent behavior.

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AT McKnight, RE Jordan, D Smart, JR Nicholson, V Brownhill & J Hughes, Parke-Davis Neuroscience Research Centre, Cambridge University Forvie Site, Robinson Way, Cambridge CB2 2QB.

The advocacy by HW Kosterlitz of the use as a screening test, of the ability of morphine and its congeners to inhibit autonomic neuroeffector transmission in *in vitro* preparations was initially the subject of scepticism, if not derision. Undaunted, the group in Aberdeen continued to develop parallel assays to measure potencies of agonists and affinity of antagonists at opioid receptors. The success of this remote unit in identifying the first endogenous agonists for opioid receptors was attributable to the use of the mouse vas deferens to bioassay extracts of brain, where their competitors, had attempted to use a binding assay. The later use in Aberdeen of the organ-bath tests, with radio-ligand binding assays, lead to the identification of the 6-receptor (Lord *et al.*, 1977), and a definitive characterisation of the K-binding site in brain (Kosterlitz *et al.*, 1981).

The use of radio-ligand binding assays, in parallel with functional assays, came to be the key step in the initiation and progression of the drug-discovery process in most of the commercial drug houses. With the advent of technologies for the identification of cDNA sequences encoding receptors, it has become the norm that the source material for primary and secondary screens, rather than animal tissue, is a cultured cell engineered to express the target molecule, in some cases with the expression of other proteins to amplify the functional response. Although there may now be a discernible trend away from the "traditional" pharmacological approach, it is still possible to apply methods of analytical pharmacology to these new "preparations". We, among others, follow measurements of affinity of novel ligands in the radio-ligand binding assay, with a rigorous determination of antagonist affinity.

Although we may use several methods in parallel to measure responses in recombinant systems, we have found the technique of microphysiometry using the Cytosensor to be particularly useful in the analyses of interactions of ligands at cloned receptors stably expressed in the CHO cell. We have used this approach with a number of G-protein-coupled receptors, and have recently found this technique is also applicable to interleukin-1 (IL-1) receptors, where the transduction mechanism is unknown.

Our first experience with the Cytosensor (Jordan *et al.*, 1994) was with CHO cells expressing the human NK3 receptor and we obtained robust and reproducible responses with serial administrations of senktide and other agonists. We characterised the receptor-effector system in terms of the relative potencies of agonists, from senktide (ECso 1.9nM) to substance P-methyl ester (ECso >311M); using standard Schild regressions we determined the affinity of antagonists with values for pA2/pKg ranging from 7.5 to 9.2, in good agreement with measurements of pKi in the same cells. The response to activation of this receptor was severely obtunded after depletion of IP3-sensitive calcium stores by thapsigargin, or by inhibition of protein kinase C by staurosporine, suggesting that the NK3 receptor was coupled to phospholipase C as was expected. While experiments in parallel with measurements of increased Cai confirmed that the response to senktide was also blocked by the phospholipase inhibitor U73122, we found that the acidification-rate response was refractory to block by this agent (Jordan *et al.*, 1995). We have no satisfactory explanation for this intriguing result, but found the same applied to the human BB1 receptor, when we went on to work with this other Gq-coupled receptor.

With the CRF receptors, traditionally functional responses have been detected by measurement of increased formation of cAMP, although the CRF2 receptor appears to be poorly coupled through Gs. By comparing the potencies of the agonists CRF, sauvagine, urocortin and urotensin we have found that the acidification-rate response of CHO-cell expressing the separate CRF receptors is an extraordinarily sensitive assay, with all four agonists equally potent at the human CRFI receptor with values for ECso between 3 and 7pM (Smart *et al.*, 1997). At the human CRF2 receptor the order of potency was urocortin (13pM) > sauvagine > urocortin > CRF (3nM), in keeping with the results of other assays. The antagonist ahelical-CRF(9-31) acted competitively at both receptors, (apparent PKB values 7.97 and 6.99) but was a partial agonist at the former type.

With the newest member of the opioid receptor family, the ORLI receptor, although the degree of structural homology towards p-, bor K-receptors is high, and there appears to exist a functional homology in that this receptor appears also to couple through Gi, there is no pharmacological homology. Our work in the Cytosensor with this receptor is only beginning, but we have been so far content to accept Nature's bounty since the brain of rats or guinea-pigs has a higher investment with the ORLI receptor than with any of the classical opioid receptor types (Paterson *et al.*, 1997), and vasa deferentia from rats and rabbits provide an extremely sensitive functional assay for nociceptin, the endogenous agonist for this receptor (Nicholson *et al.*, 1996). Most recently we have shown that 80% of neurones in the ventromedial nucleus of the hypothalamus are powerfully hyperpolarised by nociceptin, and in keeping with this effect iov administration of nociceptin increases food intake in satiated rats (Nicholson, Lee & McKnight, this meeting).

Lastly, having obtained the human IL- I type I receptor stably expressed in CHO cells we were interested to test the performance of these cells in the Cytosensor, since the pharmacology of this receptor is poorly described We found a robust acidification-rate response with the agonist IL-la, but the characteristics of the effect were different from those with G-protein-coupled receptors, since the response was slow in onset and development, and outlived the period of exposure to the agonist. Construction of the full concentration-response relationship was problematic due to desensensitisation, however we determined the ECso for IL-la as 0.1 nM, with IL- I D of comparable potency. Measurement of antagonist affinity with IL-lra was also difficult, but we have found that the response to 10ng ml~l IL-la is blocked by IL-lra in a concentration-dependent manner with an ICso of 20ng ml~l (Smart *et al*., 1997).

In conclusion, we find that we are well served in our determination to adopt a rigorous pharmacological to recombinant receptor systems by the technique of microphysiometry using the Cytosensor. The technique is applicable to receptors coupling through G-proteins to various effectors, and to at least one receptor that is not so coupled.

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456P P2X RECEPTORS: A THIRD FAMILY OF LIGAND-GATED ION CHANNELS

R.A. North, Geneva Biomedical Research Institute, Glaxo Wellcome, Plan-les-Ouates, 1228 Geneva, Switzerland

Adenosine 5'-triphosphate and/or related nucleotides act at bothionotropic (P2X) and metabotropic (P2Y) receptors. P2X receptor subunits (P2X1–P2X7) are encoded by distinct genes, having ten to twelve exons. They form ligand-gatedcation channels, either as homo-multimers or heteromultimers. P2X subunits are expressed by some smooth muscle and ventricular myocytes; in certain arterioles, ATP is the main sympathetic neurotransmitter. P2X4 subunits are expressed by several exocrine glands; together with P2X6 subunits, they are also widespread in the central nervous system. All the subunits are found in primary afferent neurons, and P2X 3 subunits participate in channels expressed by nociceptive sensory neurons.

P2X7 subunits are expressed by macrophages and brain microglia; they are unique in that the application of the agonist not only opens a cation channel (about 0.8 nm) but also leads to the development of a large pore (about 4 nm). P2X receptor subunits have intracellular N- and C-termini, with most of the protein forming a glycoslyated extracellular loop; cysteine scanning mutagenesis indicates that the second of the two transmembrane domains of each subunit contributes to the ion permeation pathway. This structure is thus fundamentally different from that of ligand-gated ion channels within the nicotinic acetylcholine or glutamate super-families; it resembles in general terms the topology of the superfamily of epithelial sodium channels/C. *elegans* degenerins, which now includes some ligand-gated members (protons/ FMRFamide).

457P THE MOLECULAR BIOLOGY OF ENDOTHELIN-CONVERTING ENZYME

Olivier Valdenaire, Preclinical Research, Pharmaceutical Division, F.Hoffmann-La Roche Ltd, Grenzacherstarsse 124, CH-4070, Basel, Switzerland.

Three related genes encode the endothelin precursors or preproendothelins. Processing of these 200 amino-acid polypeptides occurs in two stages. Dibasic amino-acid endopeptidase and carboxypeptidase activities first release the inactive big endothelins (40 residues). Big endothelins are then further cleaved at Trp-Val (Trp-Iso for big ET-3) bond to generate endothelins.

Very early it was suggested that this second step of the endothelin synthesis was mainly achieved by a specific "endothelin-converting enzyme" (ECE). This enzyme was termed ECE-1. Cloning also revealed the existence of ECE-2, a closely related enzyme sharing the same ability to convert big endothelins into endothelins. However, the acidic pH optimum and the low expression levels of ECE-2 favor the hypothesis that ECE-1 is the main enzyme involved in the endothelin conversion.

The gene encoding ECE-1 has been characterized: it encompasses at least 70kB and 20 exons, and is localised on human chromosome 1 (1p36). The organization of its 5'regions is extremely complex due to the presence of 3 alternate promoters. A consequence of this complexity is the existence of three ECE-1 isoenzymes which display divergent N-terminal extremities. When expressed in CHO cells, these three ECE-1 isoforms process big endothelins with similar efficiencies but display different intracellular localizarions.

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