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CO-EXPRESSION OF THE P75 NEUROTROPHIN RECEPTOR AND NEUROTROPHIN RECEPTOR-INTERACTING MELANOMA ANTIGEN HOMOLOG IN THE MATURE RAT BRAIN

G. L. BARRETT,[a]* U. GREFERATH,[a] P. A. BARKER,[b] J. TRIEU[a] AND A. BENNIE[a]

[a]*Department of Physiology, University of Melbourne, Parkville 3010, Australia*

[b]*Centre for Neuronal Survival, Montreal Neurological Institute, McGill University, 3801 University Avenue, Montreal, Quebec, Canada H3A 2B4*

Abstract—The p75 neurotrophin receptor ($p75^{NTR}$) is involved in the regulation of neuronal survival and phenotype, but its signal transduction mechanisms are poorly understood. Recent evidence has implicated the cytoplasmic protein NRAGE (neurotrophin receptor-interacting MAGE (from Melanoma AntiGEn) homolog) in $p75^{NTR}$ signaling. To gain further insight into the role of NRAGE, we investigated the co-expression of NRAGE and $p75^{NTR}$ in mature rat brain. In all areas examined, NRAGE appeared to be confined to neurons. In the basal forebrain cholinergic complex, NRAGE immunoreactivity was evident in all $p75^{NTR}$-positive neurons. There were many more NRAGE-positive than $p75^{NTR}$-positive neurons in these regions, however. NRAGE was also expressed in areas of the basal forebrain that did not express $p75^{NTR}$, including the lateral septal nucleus and the nucleus accumbens. A finding in marked contrast to previous studies was the presence of $p75^{NTR}$ immunoreactivity in neuronal cell bodies in the hippocampus. Hippocampal $p75^{NTR}$ immunoreactivity was apparent in rats 6 months and older, and was localized to the dentate gyrus and stratum oriens. All $p75^{NTR}$-positive neurons in the dentate gyrus and hippocampal formation were positive for NRAGE. The majority of granular cells of the dentate gyrus and pyramidal cells in the hippocampal formation were positive for NRAGE and negative for $p75^{NTR}$. NRAGE was also present in some neuronal populations that express $p75^{NTR}$ after injury, including striatal cholinergic interneurons, and motor neurons. A region of marked disparity was the cerebral cortex, in which NRAGE immunoreactivity was widespread whereas $p75^{NTR}$ was absent. The results are consistent with an important role for NRAGE in $p75^{NTR}$ signaling, as all cells that expressed $p75^{NTR}$ also expressed NRAGE. The wider distribution of NRAGE expression suggests that NRAGE may also participate in other signaling processes.

Key words: apoptosis, basal forebrain, cholinergic neuron, hippocampus.

The p75 neurotrophin receptor ($p75^{NTR}$) binds with equal affinity to each of the four classical neurotrophins, and participates in several signaling processes with diverse cellular effects. It is thought to act as an auxiliary to TrkA in the transduction of survival and differentiation signals by nerve growth factor (Meakin and Shooter, 1992). It may also co-operate with TrkB and TrkC to transduce survival and differentiation signals by other members of the neurotrophin family (Hantzopoulos et al., 1994). In certain circumstances, and particularly in the absence of TrkA, $p75^{NTR}$ can induce apoptosis (Barrett and Bartlett, 1994; Casaccia-Bonnefil et al., 1996; Frade et al., 1996; Rabizadeh et al., 1993). In cholinergic forebrain neurons, it may be responsible for an inhibitory effect on cell growth (Greferath et al., 2000a; Yeo et al., 1997). Further evidence for the multiplicity of actions of $p75^{NTR}$ was obtained more recently, with the finding that it can act as a co-receptor for Nogo (Wang et al., 2002; Wong et al., 2002). An outstanding problem in neurotrophin research is that, despite the expanding evidence for the important roles played by $p75^{NTR}$, very little is known about its signal transduction mechanisms.

Several proteins are capable of binding to, or interacting with, the intracellular domain of $p75^{NTR}$. These proteins are considered to be putative $p75^{NTR}$ signal transduction factors. The number of such proteins is steadily increasing, leading to a large number of potential $p75^{NTR}$ signaling complexes. It remains as a major challenge to determine, for each of these, the tissues, cell-types and physiological circumstances in which their interactions with $p75^{NTR}$ are manifest *in vivo*.

The MAGE (from Melanoma AntiGEn) homologue NRAGE (neurotrophin receptor-interacting MAGE homolog) was identified as a binding partner for $p75^{NTR}$ by the yeast two-hybrid assay, and has been shown to be capable of interacting with $p75^{NTR}$ under physiological conditions (Salehi et al., 2000b). As a putative signal transduction partner for $p75^{NTR}$, NRAGE is particularly interesting for several reasons. Firstly, transfection of NRAGE led to cell death in sympathetic precursor neurons, but only in the presence of $p75^{NTR}$. Secondly, the binding of NRAGE and TrkA to $p75^{NTR}$ was mutually exclusive, presenting a possible explanation for the ability of $p75^{NTR}$ to participate in both survival and apoptotic signaling. In this scenario, the absence of TrkA would allow $p75^{NTR}$ to bind to NRAGE and initiate cell death. In the presence of TrkA, however, binding of NRAGE to $p75^{NTR}$ would be blocked. $P75^{NTR}$ would instead associate with TrkA, leading to augmentation of survival signaling (although the mechanism of this

*Corresponding author. Tel: +61-3-8344-5869; fax: +61-3-8344-5818.
E-mail address: g.barrett@physiology.unimelb.edu.au (G. Barrett).
Abbreviations: HDB, horizontal limb of the diagonal band of Broca; IgG, immunoglobulin G; MAGE, Melanoma AntiGEn; NRAGE, neurotrophin receptor-interacting melanoma antigen homolog; PBS, phosphate-buffered saline; PFA, paraformaldehyde; $p75^{NTR}$, p75 neurotrophin receptor; VDB, vertical limb of the diagonal band of Broca.

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augmentation remains poorly understood). The third reason for our interest in NRAGE was that NRAGE is co-expressed with $p75^{NTR}$ in the developing brain (Kendall et al., 2002; Salehi et al., 2000a). Further interest in NRAGE has been generated by reports that it can utilize the JNK pathway to activate caspases and initiate apoptosis (Salehi et al., 2002), and that it facilitates breakdown of IAP proteins, which are inhibitors of apoptosis (Jordan et al., 2001).

We decided to investigate the $p75^{NTR}$-interacting role of NRAGE further, by investigating its expression in the mature brain and looking for further evidence of co-localization with $p75^{NTR}$. The aims of the present study were to look for evidence of NRAGE expression in neurons that express $p75^{NTR}$, and to assess the degree to which the two proteins are co-expressed. The underlying rationale was that the degree of functional interaction between $p75^{NTR}$ and NRAGE would be reflected in the extent to which they are expressed in the same cells. A recent report has described widespread expression of NRAGE in the developing and adult brain, but did not look specifically at the issue of co-expression with $p75^{NTR}$ (Kendall et al., 2002). If NRAGE is an important factor in $p75^{NTR}$ signal transduction, it would be expected to be expressed in $p75^{NTR}$-expressing cells. On the other hand, lack of co-expression at the cellular level would not be consistent with a physiological interaction.

The pattern of expression of $p75^{NTR}$ in the mature brain is very distinctive: strong expression, in non-pathological circumstances, is largely confined to cholinergic neurons of the basal forebrain complex (Richardson et al., 1986). This complex entails several contiguous anatomical identities, including the medial septum, vertical and horizontal limbs of the diagonal band of Broca (VDB and HDB, respectively) and the basal nucleus of Meynert. There is very nearly a one-to-one correspondence between the expression of cholinergic markers and $p75^{NTR}$ in neurons in these regions (Greferath et al., 2000b).

In addition to testing the hypothesis that NRAGE is co-localized with $p75^{NTR}$ in the mature nervous system, we decided to re-examine some aspects of $p75^{NTR}$ expression. Although its expression in the basal forebrain nuclei has been thoroughly documented, the expression of $p75^{NTR}$ elsewhere in the brain remains the subject of conflicting reports. In the hippocampus, electron microscopic studies have localized $p75^{NTR}$ to afferent axons and presynaptic terminals (Dougherty and Milner, 1999; Kawaja and Gage, 1991). Only a small amount of $p75^{NTR}$ was found in neuronal bodies, and this was within endosomes and thus presumably due to endocytosis. However, one study described abundant $p75^{NTR}$ in neuronal cell bodies of the polymorph layer of the dentate gyrus and CA3 pyramidal layer, but only after administration of the axonal transport inhibitor colchicine (Pioro and Cuello, 1990).

Considerable disagreement also exists about the expression of $p75^{NTR}$ in the adult striatum. Some studies have found a small number of weakly $p75^{NTR}$-positive medium-sized neurons in the striatum, and these were thought to be cholinergic interneurons (Kiss et al., 1988; Pioro and Cuello, 1990). Other studies have reported an absence of $p75^{NTR}$ expression in the striatum (Gage et al., 1989; Lee et al., 1998). There is also some disagreement about the complete absence of $p75^{NTR}$ from cortical neurons. In general, surveys of the adult rat brain have failed to detect $p75^{NTR}$-immunoreactive neurons, although abundant $p75^{NTR}$-positive cells were found in one study of the cortex in mature rats (Miller and Pitts, 2000).

The significant discrepancies in reports of $p75^{NTR}$ in the hippocampus, striatum and cortex may reflect very low levels of expression, levels that are hard to distinguish from background staining. Another possible factor relates to different ages of the animals studied. In general, studies on mature rats have described the weights of the animals studied, but not their exact ages. Most studies in which the group of animals are categorised by weight are likely to include a mixture of different ages, with a likely bias toward relatively young animals. In anticipation of possible age-related variations, we therefore surveyed $p75^{NTR}$ and NRAGE expression in rats of various ages. We confined our study to areas of the CNS reported to express $p75^{NTR}$ in postnatal animals. We examined in particular the basal forebrain, hippocampus, striatum, cerebral cortex and motor nuclei.

EXPERIMENTAL PROCEDURES

Animals and treatment

Rats of the Sprague–Dawley and Dark Agouti strains, of both sexes, were used. Sprague–Dawley rats were obtained from the Walter and Eliza Hall Animal Facility (Melbourne, Australia), and Dark Agouti rats from the Australian Research Council Animal Facility (Adelaide, Australia). Rats were housed in groups of two or three on a 12-h light/dark cycle with food and water available *ad libitum*. Animals aged 1, 3, 6, 12, 17, and 26 months were killed and perfused, and their brains were removed. Euthanasia was by overdose of Nembutal (125 mg/kg) administered intraperitoneally. Rat weights were between 90 and 125 g (1-month-old) and 150 and 320 g (6 months and older) at the time they were killed. Two-month-old mice of the 129/Sv strain were used for some experiments.

Throughout the period of their housing, animals were monitored daily for general health and their weights were measured at regular intervals. The experiments and mode of killing were approved by the University of Melbourne Animal Experimentation Ethics Committee, in accordance with guidelines established by the National Health and Medical Research Council of Australia. These guidelines are in accordance with accepted international guidelines on the ethical use of animals. Care was taken to minimize the number of animals used and their suffering.

Tissue collection

Fixation perfusion was performed after the animals were killed by Nembutal overdose. Phosphate-buffered saline (PBS, 100 ml) was administered transcardially, followed by 100 ml of 4% paraformaldehyde (PFA) in PBS. Brains were then removed and post-fixed overnight in 4% PFA, followed by 24 h incubation in 30% sucrose in PBS. Brains were stored at −20 °C in cryoprotectant (15% sucrose, 15% ethylene glycol) until sectioned. Serial coronal sections of the basal forebrain (medial septum, VDB and HDB) were taken from the genu of the corpus callosum rostrally to the optic chiasm caudally. These sections were also used to examine the cerebral cortex and caudate putamen. To visualize the hippocampus, coronal sections were taken through the caudal part of