

The Development of Differential mabQ113-Immunoreactivity in the Rat Habenular Complex

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PLIOPLYS, A. V. AND R. HAWKES. *The development of differential mabQ113-immunoreactivity in the rat habenular complex*. BRAIN RES BULL 18(1) 19–24, 1987.—Monoclonal antibody mabQ113 selectively labels a subset of Purkinje cells which are arranged in parasagittal bands throughout the vermis and hemispheres of the rat cerebellar cortex. No other cerebellar cell types are immunoreactive. By contrast, in the remainder of the brain the mabQ113 epitope is located primarily in glial cells. In general, the glial immunoreactivity is not differentially distributed. An exception is that mabQ113 densely and uniformly stains the lateral habenula (LHb) but gives no labelling of the medial habenula (MHb). During cerebellar development, the mabQ113 epitope is expressed in three stages. Before postnatal day 7 (P7) all Purkinje cells are negative. Secondly, all Purkinje cells become mabQ113+ between P7 and P12. The parasagittal bands are created between P12 and P30 by selective suppression of epitope expression. To explore whether epitope suppression is also responsible for differential staining patterns in other brain regions the ontogenic development of mabQ113 immunoreactivity has been mapped in the habenular complex. Neither the MHb nor the LHb express the mabQ113 epitope prenatally. P1 is the first age at which the LHb is stained. During the next few days the intensity of staining within the LHb steadily increases until the adult pattern is attained at P6. At no time is there expression of the mabQ113 antigen in the MHb. This also confirms that the two classes of habenular astrocytes, mabQ113–/GFAP+ and mabQ113+/GFAP+, are intrinsically different throughout postnatal life.

Development Habenula Immunocytochemistry Monoclonal antibodies

AS a crossroads of limbic and striatal interconnections the habenular nuclei play a role in numerous aspects of brain function (reviewed in [38]). Although grouped together, the medial (MHb) and lateral (LHb) habenular nuclei have widespread, largely independent afferent and efferent connections (for the rat see [6, 7, 11, 12, 19, 20, 25, 34, 39]). Numerous biochemical differences between LHb and MHb have also been reported, involving components of the GABAergic [9, 10, 40] and cholinergic [13, 22, 23, 29, 32] pathways as well as several other neurotransmitters [2, 10, 20, 24] and neuropeptides [1, 5, 8, 21, 28, 34, 36, 37]. Recently, a monoclonal antibody, mabQ113, has been found to differentiate sharply between LHb and MHb. The mabQ113 epitope is of especial interest because in the cerebellum it is confined to a subset of Purkinje cells, about 30% of the total, which are clustered into a set of parasagittal bands separated by similar bands of mabQ113–Purkinje cells [15–18, 30]. No other cells in the cerebellum are immunoreactive. By contrast, when mabQ113 immunoreactivity was mapped in the rest of the rat CNS the distribution of reaction product was quite different. In all regions studied, the epitope was found in both neurons and glia, but principally in glial cells, and there was no evi-

dence of a striped or patchy distribution [31]. One region in which the epitope was distributed non-uniformly was the habenular complex. MabQ113 densely and uniformly stained the LHb but did not stain the MHb. The reaction product was associated with the neuropil and electron microscope immunocytochemistry revealed that most staining was astrocytic, although some reaction product was also seen in adjacent neuronal profiles. Therefore, there seems to be a fundamental difference between the expression of the epitope in the cerebellum and the rest of the brain.

In the cerebellum the pattern of development of parasagittal mabQ113+ bands of Purkinje cells is complex. The mabQ113 immunoreactivity appears for the first time at postnatal day 7 (P7) in the Purkinje cells of the posterior lobe vermis. By P12, the immunoreactivity has spread to include Purkinje cells throughout the cerebellar cortex. However, at P12 there is no differentiation between mabQ113+ and mabQ113– bands of cells: all the Purkinje cells are mabQ113+. The parasagittal bands are created between P12 and P30 by the selective suppression of epitope expression in those Purkinje cells destined to become mabQ113– [16]. In view of this pattern of development in the cerebellar cortex,

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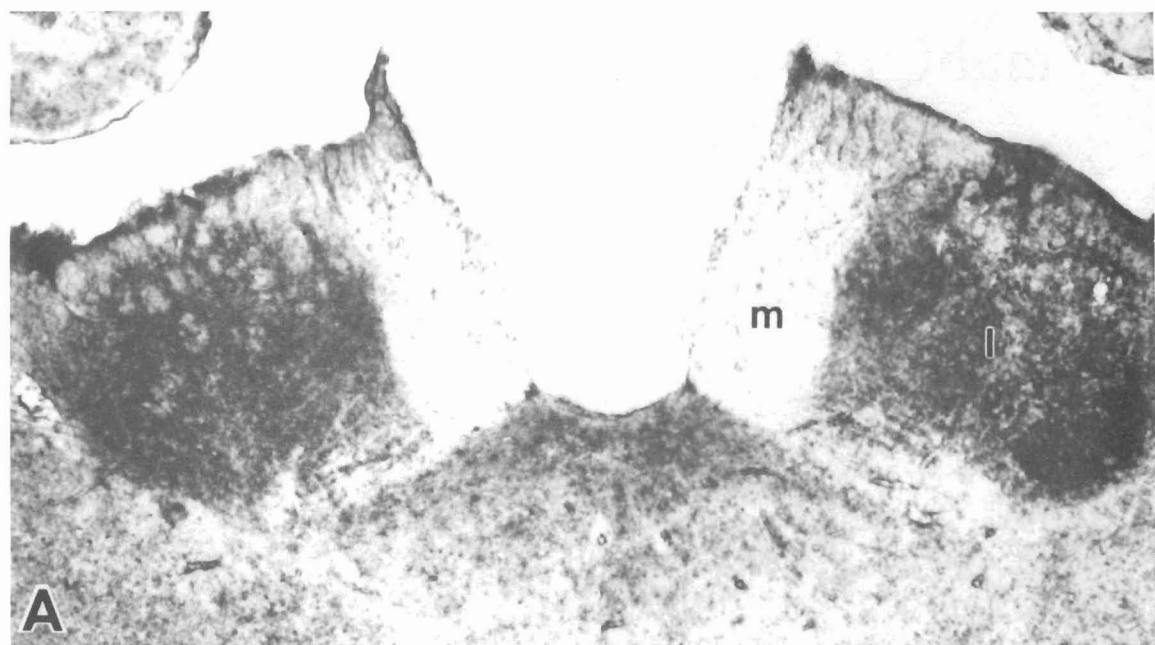


FIG. 1. Coronal sections of adult rat brain immunoperoxidase stained with mabQ113 (A) and anti-glial fibrillary acidic protein (GFAP) (B). A. MabQ113 densely stains the lateral habenula (l) and leaves the medial habenula (m) unstained. The reaction product is distributed throughout the neuropil of the LHb with no systematic regional differences in intensity. B. Anti-GFAP stains the astrocytes of both the m and l equally. The scale bar indicates 200 μ m.

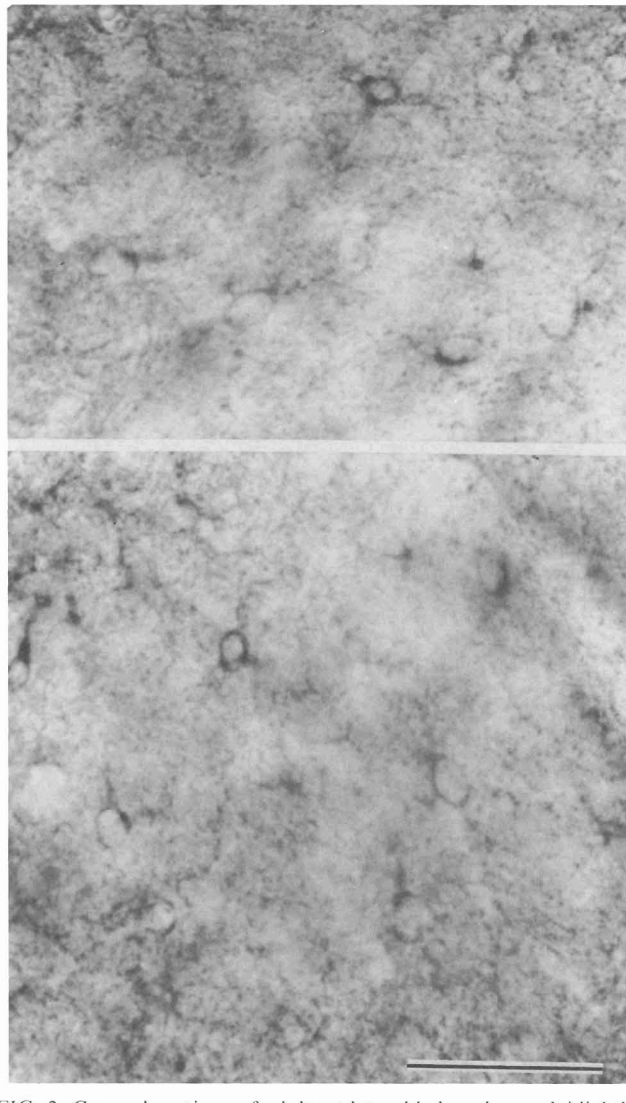


FIG. 2. Coronal sections of adult rat lateral habenular nuclei lightly immunoperoxidase stained with mabQ113. The cell bodies of small multipolar cells can be distinguished. The somata range in diameter from 7 to 14 μm . The scale bar indicates 50 μm .

it was important to discover whether selective suppression of epitope expression was also responsible for the non-uniform distribution of mabQ113 immunoreactivity in other brain regions, where the reaction product is primarily associated with astroglia. Therefore, we have tested whether differential staining in the habenular complex develops through the selective suppression of epitope expression in a subset of astroglia or whether two astroglial populations are distinct from the earliest stages of expression.

METHOD

The production and characterization of monoclonal antibody mabQ113 has been described previously [15]. To obtain adult tissue for immunocytochemistry, rats were first

deeply anesthetized with sodium pentobarbital. After surgical exposure of the heart, 75 units of heparin and 5 mg sodium nitrite were injected into the heart and, one minute later, the animal was perfused via the left ventricle with 250 ml of

ice-cold fixative (4% paraformaldehyde, 0.2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4) over a 30 min period. The brains were removed and either placed in phosphate buffered saline (0.1 M phosphate buffer, 0.15 M NaCl, pH 7.4) or postfixed overnight in 4% paraformaldehyde in phosphate buffer alone.

For the developmental series, pregnant rats and postnatal pups were deeply anesthetized with sodium pentobarbital. Fetuses were taken at gestational ages E15, E17 and E20, the day of copulation being E0. The fetuses were removed surgically through an incision in the uterine wall. E17 and E20 fetuses and postnatal pups were perfused over a 15 min period via the left ventricle with ice-cold fixative with a volume ranging from 2 to 30 ml depending on the size of the animal. E15 fetuses were too small for reliable intracardiac perfusion. After removal from the uterine sack, they were decapitated and their heads promptly immersed in cold fixative for periods of time ranging from 15 min to 24 hr. Complete litters (8–12 individuals) were fixed at each prenatal age and five individuals, from different litters, were taken at each postnatal age. The brains of the immersion-fixed and perfused fetuses and postnatal rats were removed and stored in phosphate buffered saline.

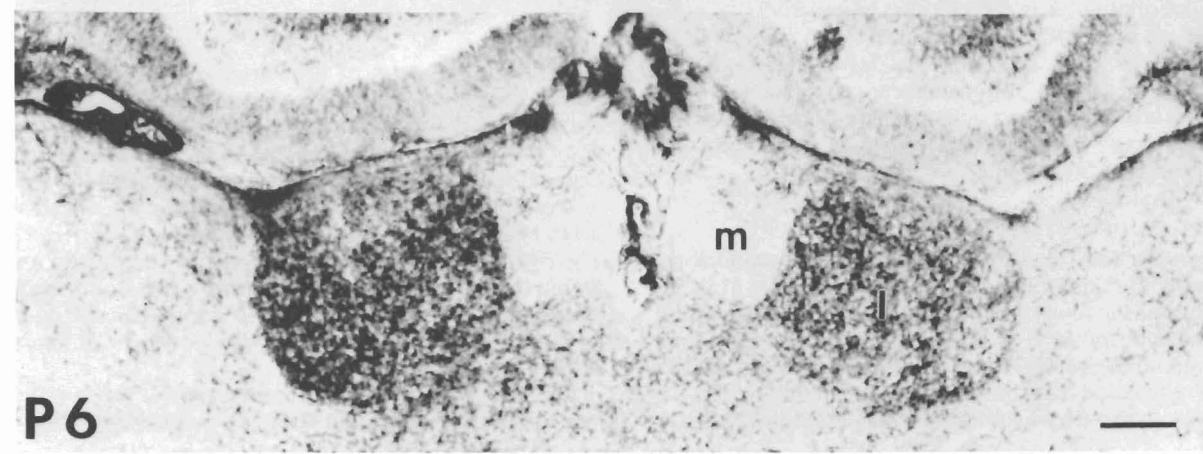
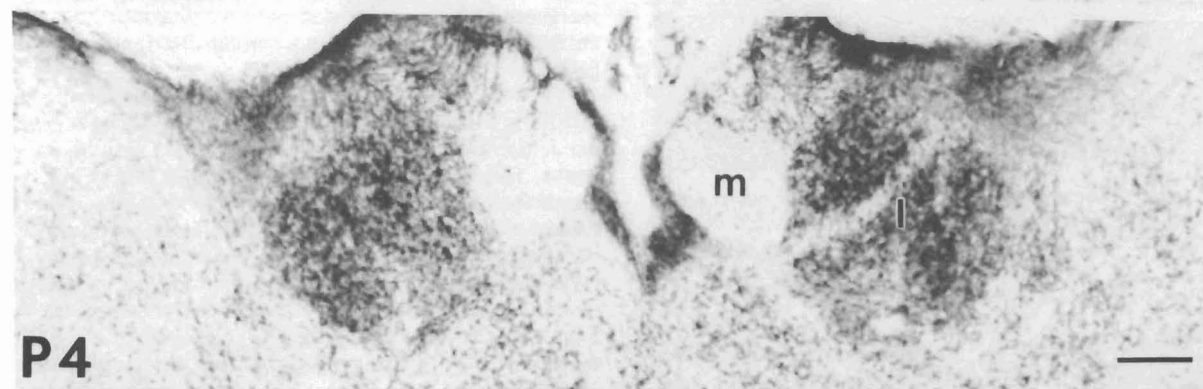
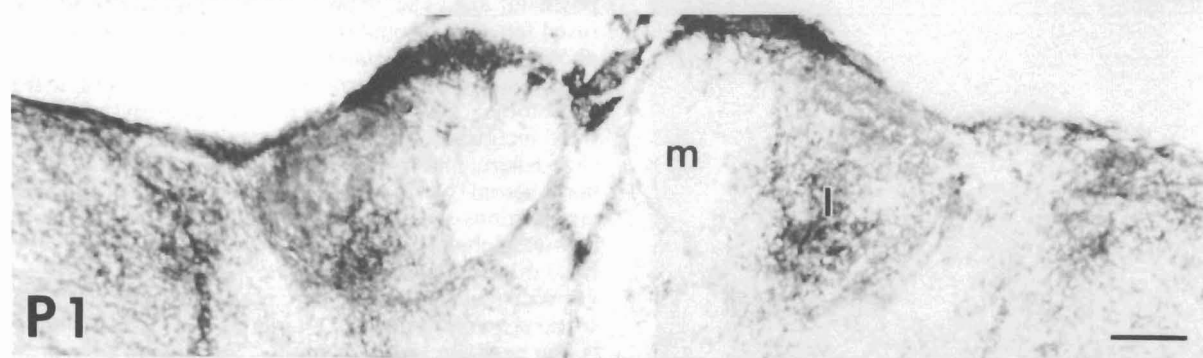
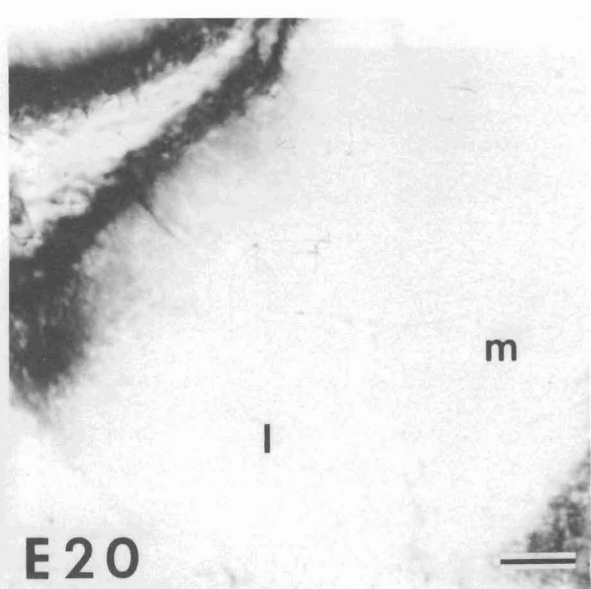
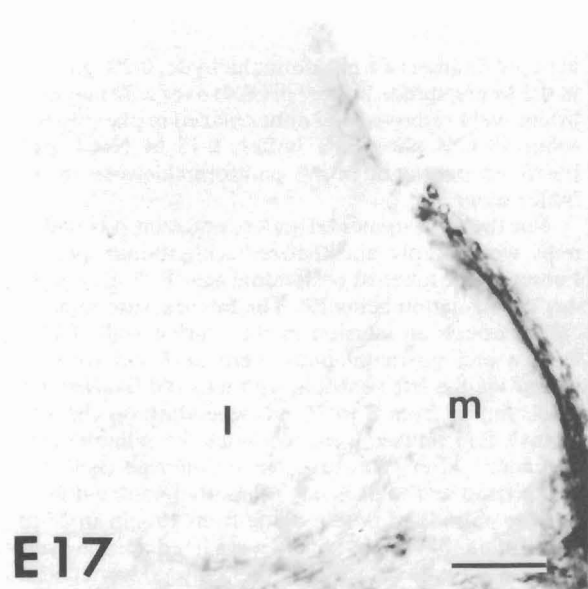
Sections were cut coronally at 50 μm using a freezing microtome. To detect specific immunoreactivity, sections were incubated in antibody overnight. In all the examples shown here, mabQ113 was used diluted 1/16 into 10% normal horse serum (NHS) in PBS. To detect specific antibody binding, sections were incubated for 2 hr in rabbit anti-mouse immunoglobulin conjugated to horseradish peroxidase (Dako Inc.) diluted 1/100 in 10% NHS. Antibody binding was revealed by using 4-chloro-1-naphthol as substrate [14]. The sections were washed for 10 min in each of three changes of buffer between the incubations. Sections in which the primary antibody was omitted gave no staining and no other antibody in our library gave a similar distribution of reaction product.

To detect the presence of glial fibrillary acidic protein (GFAP), a polyclonal rabbit anti-mouse GFAP serum was used, the kind gift of Dr. V. I. Kalnins, University of Toronto. The serum was diluted 1/20 in 10% NHS. The remainder of the histochemical procedures were as described above except that goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase (Sigma Inc.) diluted 1/100 in 10% NHS was used. To confirm the specificity of the anti-GFAP reagent these experiments were repeated using the Histo-Scan anti-GFAP staining kit (Biomedica Corp.). The results were identical by using either staining procedure.

RESULTS

With the exception of the Purkinje cells of the cerebellar cortex, mabQ113 immunoreactivity in the adult rat brain is strongest in the habenular complex (Fig. 1A). In the Lhb, mabQ113 immunocytochemistry leads to a dense, uniform deposition of peroxidase reaction product which is confined to the tangle of processes in the neuropil. By contrast, the MHb is unreactive except for an occasional scattered fiber. The boundary between Lhb and MHb is sharply defined and within the Lhb there is no evidence of a non-uniform epitope distribution. Previous studies have shown that the mabQ113 immunoreactivity in the neuropil is localized primarily to astroglial processes [31].

GFAP is a reliable immunocytochemical marker of astrocytes in the mammalian central nervous system [3,4]. Using



anti-GFAP reagents both the LHb and MHb are uniformly stained (Fig. 1B), thus indicating that the selective distribution of the mabQ113 epitope is not due to a lack of astrocytes in the MHb. Furthermore, this observation confirms the presence of two classes of astrocytes mabQ113+/GFAP+ and mabQ113-/GFAP+ as has been shown in the rat cerebral cortex [31]. The MHb is populated by mabQ113-/GFAP+ astrocytes and the LHb by mabQ113+/GFAP+ ones.

The mabQ113 staining of the LHb is so intense that individual stained cellular elements usually cannot be distinguished, and only the outlines of large unstained neurons are identifiable [31]. In lightly stained sections it is apparent that reaction product is associated with small cells bearing numerous fine processes (Fig. 2). The diameter of the cell bodies ranges between 7 and 14 μm with the majority being 11–12 μm in size.

The development of mabQ113 immunoreactivity of the habenular complex has been investigated from embryonic day 15 (E15) onwards. There is no specific immunoreactivity in either the LHb or the MHb in the prenatal rat (Fig. 3). This is not the case for other brain regions: for example, in the telencephalic vesicle at E17, the ventricular zone and radial glial fibers are all strongly mabQ113-reactive [31]. Immunoreactive neuropil first appears in the LHb during the first day after birth (P1; Fig. 3). Even at the earliest stages of induction, there is no evidence for a gradient of expression within the LHb. During the next few days the intensity of staining within the LHb steadily increases until the adult level of immunoreactivity is attained at P6 (Fig. 3). At no stage during development is the mabQ113 epitope expressed by cells of the MHb.

DISCUSSION

By using tritiated thymidine autoradiographic techniques, it has been found that the cells of the habenular nuclei are generated between E11 and E15, being slightly more advanced in the LHb as compared to the MHb (R. Marchand, personal communication), and by cresyl violet staining the habenular complex is anatomically divisible into LHb and MHb nuclei between E15 and E16. The habenular afferent and efferent tracts, the stria medularis and fasciculus retroflexus, are already anatomically identifiable structures in close apposition to the habenular nuclei at E14 (R. Marchand, personal communication). The timing of maturation of afferent and efferent synaptic contacts of the habenular

nuclei have not been reported. Although numerous biochemical differences exist between the MHb and LHb, there have been few investigations into the developmental profiles of differentiation markers within the habenular complex. One exception is somatostatin-like immunoreactivity. Somatostatin-immunoreactive cells appear in the rat hypothalamus at E14 and by E20 are identifiable in the LHb [35]. Thus the appearance of mabQ113 LHb reactivity at P1 does not correspond to the time of anatomical subdivision into the LHb and MHb.

Since the peroxidase reaction product in the habenular complex is located primarily in astrocytes [31], it is clear that mabQ113 distinguishes between two populations of GFAP+ astrocytes, one mabQ113+ found in the LHb, the other mabQ113- found in the MHb. Similar evidence for two classes of astrocyte has also been obtained from the cerebral cortex [31]. Other immunological studies have demonstrated convincingly that there are at least two populations of fibrous astrocytes in the developing rat brain. In the optic nerve, type-1 astrocytes can be distinguished from type-2 by the presence or absence of galactocerebroside and there is both direct and indirect evidence that the two types develop from distinct precursor cells [26,27] and have different developmental timetables. The difference between mabQ113+ and mabQ113- astrocytes does not seem to correspond to the type-1/type-2 distinction. Likewise, the two classes of astrocytes recognized by Schachner and colleagues [33] also seem to be different from those recognized by mabQ113.

The developmental profile of mabQ113 immunoreactivity in the habenular complex has revealed another difference between the expression of the epitope in the cerebellum versus the rest of the brain, as it is clear that there is no selective suppression of immunoreactivity in the MHb equivalent to that leading to the adult mabQ113-Purkinje cells. At no stage during development is the mabQ113 epitope expressed in the MHb. Furthermore, the timing of antigen induction in the habenular complex now shows that the two glial populations are already distinct shortly after the time of birth.

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FIG. 3. Coronal sections of fetal and postnatal rat brains immunoperoxidase stained with mabQ113. At E17 and E20 there is no specific staining of either the lateral (l) or medial (m) habenula. P1 is the first age at which specific immunoreactivity is detected in the LHb. The intensity of staining within the LHb steadily increases during the next few days to attain the adult level at P6. At no age is there any specific immunoreactivity in the MHb. The scale bars indicate 50 μm for E17 and E20, and 100 μm for P1 through P6.

REFERENCES

- Barry, J. Septo-epithalamo-habenular LRF reactive neurons in monkeys. *Brain Res* **151**: 183-187, 1978.
- Beckstead, R. M., V. B. Domesick and W. J. H. Nauta. Efferent connections of the substantia nigra and ventral tegmental area in the rat. *Brain Res* **175**: 191-217, 1979.
- Bignami, A. and D. Dahl. Differentiation of astrocytes in the cerebellar cortex and the pyramidal tracts of the newborn rat. An immunofluorescence study with antibodies to a protein specific to astrocytes. *Brain Res* **49**: 393-402, 1973.
- Bignami, A. and D. Dahl. Astrocyte-specific protein and neuroglial differentiation. An immunofluorescence study with antibodies to the glial fibrillary acidic protein. *J Comp Neurol* **153**: 26-38, 1974.
- Buijs, R. M. Intra- and extrahypothalamic vasopressin and oxytocin pathways in the cat. *Cell Tissue Res* **192**: 432-435, 1978.
- Conrad, L. C. A. and D. W. Pfaff. Efferents from medial basal forebrain and hypothalamus in the rat. II. An autoradiographic study of the anterior hypothalamus. *J Comp Neurol* **169**: 221-262, 1976.
- Contestabile, A. and B. A. Flumerfelt. Afferent connections of the interpeduncular nucleus and the topographic organization of the habenulo-interpeduncular pathway: an HRP study in the rat. *J Comp Neurol* **196**: 243-270, 1981.
- Cuello, A. C., P. E. Emson, G. Paxinos and T. Jessel. Substance P containing and cholinergic projections from the habenula. *Brain Res* **149**: 413-429, 1978.
- Gottesfeld, Z., C. Brandon and J.-Y. Wu. Immunocytochemistry of glutamate decarboxylase in the deafferented habenula. *Brain Res* **208**: 181-186, 1981.
- Gottesfeld, Z. and D. M. Jacobowitz. Further evidence for GABAergic afferents to the lateral habenula. *Brain Res* **152**: 609-613, 1978.
- Greatrex, R. M. and O. T. Phillipson. Demonstration of synaptic input from prefrontal cortex to the habenula in the rat. *Brain Res* **238**: 192-197, 1982.
- Hamilton, L. W. *Basic Limbic System Anatomy of the Rat*. New York: Plenum Press, 1976, pp. 63-72.
- Hattori, T., E. G. McGeer, V. K. Singh and P. L. McGeer. Cholinergic synapses of the interpeduncular nucleus. *Exp Neurol* **55**: 666-679, 1977.
- Hawkes, R., E. Niday and J. Gordon. A dot immunobinding assay for monoclonal and other antibodies. *Anal Biochem* **119**: 142-147, 1982.
- Hawkes, R., M. Colonnier and N. Leclerc. Monoclonal antibodies reveal sagittal banding in the rodent cerebellar cortex. *Brain Res* **333**: 359-365, 1985.
- Hawkes, R. and N. Leclerc. The postnatal development of antigenic sagittal bands in the cerebellar cortex by selective repression of immunoreactivity. *Soc Neurosci Abstr* **10**: 991, 1985.
- Hawkes, R. and N. Leclerc. Immunocytochemical demonstration of topographic ordering of Purkinje cell axon terminals in the fastigial nuclei of the rat. *J Comp Neurol* **244**: 481-491, 1986.
- Hawkes, R. and N. Leclerc. An antigenic map of the rat cerebellar cortex: the distribution of parasagittal bands as revealed by monoclonal anti-Purkinje cell antibody mabQ113. *J Comp Neurol*, in press, 1986.
- Herkenham, M. and W. J. H. Nauta. Afferent connections of the habenular nuclei in the rat. A horseradish peroxidase study, with a note on the fiber-of-passage problem. *J Comp Neurol* **173**: 123-146, 1977.
- Herkenham, M. and W. J. H. Nauta. Efferent connections of the habenula nuclei in the rat. *J Comp Neurol* **187**: 19-48, 1979.
- Hokfelt, T., J. O. Keller, G. Nilsson and W. Pernow. Substance P localization in the central nervous system and in some primary sensory neurons. *Science* **190**: 889-890, 1975.
- Hoover, D., E. A. Muth and D. M. Jacobowitz. A mapping of the distribution of acetylcholine, choline acetyltransferase and acetylcholinesterase in discrete areas of rat brain. *Brain Res* **153**: 295-306, 1978.
- Hunt, S. and J. Schmidt. Some observations on the binding patterns of α -bungarotoxin in the central nervous system of the rat. *Brain Res* **157**: 213-232, 1978.
- Kuhar, M. J., G. K. Aghajanian and R. H. Roth. Tryptophan hydroxylase activity and synaptosomal uptake of serotonin in discrete brain regions after midbrain raphe lesions: correlations with serotonin levels and histochemical fluorescence. *Brain Res* **44**: 165-176, 1972.
- Marchand, E. R., J. N. Riley and R. Y. Moore. Interpeduncular nucleus afferents in the rat. *Brain Res* **193**: 339-352, 1980.
- Miller, R. H. and M. C. Raff. Fibrous and protoplasmic astrocytes are biochemically and developmentally distinct. *J Neurosci* **4**: 585-592, 1984.
- Miller, R. H., S. David, R. Patel, E. R. Abney and M. C. Raff. A quantitative immunohistochemical study of macroglial cell development in the rat optic nerve: in vivo evidence for two distinct astrocyte lineages. *Dev Biol* **111**: 35-41, 1985.
- Neckers, L. M., J. D. Schwartz, R. J. Wyatt and S. G. Speciale. Substance P afferents from the habenula innervate the dorsal raphe nucleus. *Exp Brain Res* **37**: 619-623, 1979.
- Parent, A. and L. L. Butcher. Organization and morphologies of acetylcholinesterase-containing neurons in the thalamus and hypothalamus of the rat. *J Comp Neurol* **170**: 205-226, 1976.
- Plioplys, A. V., J. Thibault and R. Hawkes. Selective staining of a subset of Purkinje cells in the human cerebellum with monoclonal antibody mabQ113. *J Neurol Sci* **70**: 245-256, 1985.
- Plioplys, A. V. and R. Hawkes. A survey of mabQ113 immunoreactivity in the adult rat brain: differential staining of the lateral and medial habenular nuclei. *Brain Res* **375**: 1-12, 1986.
- Rotter, A., N. J. M. Birdsall, A. S. V. Burgen, P. M. Field, E. C. Hulme and G. Raisman. Muscarinic receptors in the central nervous system of the rat. I. Technique for autoradiographic localization of the binding of 3H-propylbenzilylcholine mustard and its distribution in the forebrain. *Brain Res Rev* **1**: 141-165, 1979.
- Schachner, M. Glial antigens and the expression of neuroglial phenotypes. *Trends Neurosci* **5**: 225-228, 1982.
- Shinoda, K., S. Inagaki, S. Shiosaka, J. Kohno and M. Tohyama. Experimental immunohistochemical studies on the substance P neuron system in the lateral habenular nucleus of the rat: distribution and origins. *J Comp Neurol* **222**: 578-588, 1984.
- Shiosaka, S., K. Takatsuki, M. Sakanaka, S. Inagaki, H. Takagi, E. Senba, Y. Kawai, H. Iida, H. Minagawa, Y. Hara, T. Matsuzaki and M. Tohyama. Ontogeny of somatostatin-containing neurons system of the rat: immunohistochemical analysis. II. Forebrain and diencephalon. *J Comp Neurol* **204**: 211-224, 1982.
- Silverman, A. J. and L. C. Krey. The luteinizing hormone-releasing (LH-RH) neuronal networks of the guinea pig brain. I. Intra- and extra-hypothalamic projections. *Brain Res* **157**: 233-246, 1978.
- Sofroniew, M. W. and A. Weindl. Projections from the parvocellular vasopressin- and neurophysin-containing neurons of the suprahypothalamic nucleus. *Am J Anat* **153**: 391-430, 1978.
- Sutherland, R. J. The dorsal diencephalic conduction system: A review of the anatomy and functions of the habenular complex. *Neurosci Biobehav Rev* **6**: 1-13, 1982.
- Swanson, L. W. and W. M. Cowan. The connections of the septal region in the rat. *J Comp Neurol* **186**: 621-656, 1979.
- Vincent, S. R., H. Kimura and E. G. McGeer. A histochemical study of GABA-transaminase in the efferents of the pallidum. *Brain Res* **241**: 162-165, 1982.