Uptake and binding of some neurotransmitter substances and their analogues in the developing cerebellum of normal rodents and neurologically mutant mice.

Thesis

UPTAKE AND BINDING OF SOME NEUROTRANSMITTER SUBSTANCES AND THEIR ANALOGUES IN THE DEVELOPING CEREBELLUM OF NORMAL RODENTS AND NEUROLOGICALLY MUTANT MICE.

A Thesis presented to The Open University in part fulfillment of the requirements for the Degree of Doctor of Philosophy.

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CONTENTS

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ABSTRACT

Cells dissociated from the postnatally developing rat cerebellum retain their high affinity carrier-mediated transport system for (^{3}H) GABA (Kt = 1.9 uM, V = 1.8 pmol per 10^6 cells per min.) and (^{3}H) glutamate (Kt = 10 uM, V = 7.9 pmol per 10^6 cells per min.). Using a unit gravity sedimentation technique it was demonstrated that (^{3}H) GABA was taken up principally into fractions which were enriched in such neuronal types as Purkinje, stellate and basket cells. (^{3}H) β-alanine (which is known to be taken up specifically by the glial GABA transport system) and (^{3}H) glutamate were concentrated by cells of the same size range. (^{3}H) glutamate uptake was minimal in fractions enriched in precursors of granule cells. These results are discussed in relation to reports of high affinity (^{3}H) glutamate uptake by glia. The role of glutamate transport in glutamatergic cells is also considered.

In addition the development of GABA, β-adrenergic and muscarinic acetylcholine receptors in the cerebella of weaver (wv), reeler, (rl) staggerer (sg) and jimpy (jp), neurological mutant mice and their normal counterparts was examined using the radioligands, (^{3}H) muscimol, (^{3}H) dihydroalprenolol ((^{3}H) DHA) and (^{3}H) quinuclidinylbenzilate ((^{3}H) QNB). The maximum increase in ligand binding occurred during the period 15-20 days for (^{3}H) muscimol, 10-15 days for (^{3}H) DHA, 5-15 days for (^{3}H) QNB. Binding of all ligands was significantly reduced with respect to controls in the cerebellar mutants, wv, rl and sg. (^{3}H) Muscimol binding was the most affected (4-19 per cent control at 20 days) and (^{3}H) QNB binding

was least affected (36-50 percent control at 20 days). The corresponding figures for (^{3}H) DHA were 14-22 percent. Binding of these ligands to jp tissue was not significantly different from control except for (^{3}H) QNB binding which was 80 percent of control at 20 days. Binding of all ligands was saturable and of high affinity (muscimol Kd = 12.8 nM; DHA Kd = 0.26 nM; QNB Kd = 0.14 nM) and the affinity constants for binding did not change significantly during development. These data are discussed in relation to (i) the known pharmacology and development of cerebellar neurons and (ii) the effects of the mutations on the development of cerebellar neurons.

ABBREVIATIONS USED

ACHC	cis-aminocyclohexanecarboxylic acid
AOAA	aminooxyacetic acid
BSA	bovine serum albumin
CF-EBSS	calcium free Earle's basic salt solution
DHA	dihydroalprenolol
DNase	deoxyribonuclease
GABA	γ-aminobutyric acid
HEPES	N-2-hydroxyethylpiperazine-N'-2 ethansulfonic acid
IC_{50}	the concentration of inhibitor required to reduce binding or uptake to 50 per cent of control
IHYP	iodohydroxybenzylpindolol
jp	jimpy mutant
nr	homozygous nervous mutant
PBCM	propylbenzilylcholine mustard
PSG	Puck's saline g
QNB	quinuclidinylbenzilate
rl	homozygous reeler mutant
SBT1	soya bean trypsin inhibitor
sg	homozygous staggerer mutant
tris	tris(hydroxymethyl) aminomethane
wv	homozygous weaver mutant

Aims

The overall aim of this thesis was to gain further information concerning the uptake of neurotransmitter candidates by various cerebellar cell types and to get some idea of the receptor populations associated with these cells. This was tackled in two separate ways. (i) A method for the isolation of cells from the developing rodent cerebellum has been developed in this laboratory along with a method for the separation of these cells on the basis of size. The high affinity transport of GABA, β-alanine and glutamate was examined in cell fractions enriched in particular neuronal types in order to further characterise the fractions, and to see whether glutamate uptake could be assigned to a particular cell type. The uptake of glutamate is of particular relevance since cerebellar granule cells are thought to use glutamate as their neurotransmitter and the uptake of glutamate by glutamatergic cells has been reported by some workers but is disputed by others (see Section 1.6). (ii) The second part of this thesis does not lead directly on from the above work because as outlined in Chapter 2 a large part of the work intended (e.g. characterisation of uptake by various cell types) had been completed by another group of workers. The final chapter of the thesis outlines work using radioligand techniques to examine the development of neurotransmitter receptors in normal and agranular mutant mouse cerebellum. The aim of this group of studies was to see whether the development of any group of receptors (i.e. GABA, β-adrenergic or muscarinic cholinergic receptors) could be linked to known synaptogenic events; and in addition to see if the development of receptors in agranular cerebella could provide clues as to the cell types on which these receptors were located. It was hoped that such studies would also tell us something about the way in which the loss of one neuronal type affects the development of the remaining receptor populations.

CHAPTER 1

INTRODUCTION

1.1 Structure and function of the cerebellum

The cerebellum is phylogenetically one of the oldest structures of the vertebrate brain. It is situated dorsal to the mid-brain, and posterior to the cerebral cortex. Superficially the cerebellum resembles the cerebrum since it has a highly convoluted appearance, and in section peripheral neuronal structures (cerebellar cortex) overlie white matter and cerebellar nuclei.

Owing to the uniform cytoarchitecture of the mature cerebellum first described by Ramon y Cajal, (1955), the cortex of the cerebellum is probably the most well characterised area of the brain. The cortex contains only five main neuronal types; Purkinje, Golgi, stellate and basket cells, all of which are inhibitory, and the granule cells which are excitatory. There are two major inputs (as revealed by classical histological techniques) the climbing fibres and mossy fibres which are both excitatory. The sole output of the cortex is the Purkinje axons which synapse with the deep cerebellar nuclei (Eccles *et al.* 1967).

Although much of the cerebellar circuitry has been characterised it is beyond the scope of this introduction to discuss the full range of interactions which are thought to take place. However,

a brief outline of the major pathways is given below. Unless cited otherwise, the information is taken from the following sources (Eccles et al. 1967; Palay & Chan Palay, 1974). The qualitative data are taken from Palkovits et al. (1971a,b,c) and Eccles(1973), and are for the cat. Although the numbers quoted below are an order of magnitude lower for the rat and mouse, the organisation is the same (Palay & Chan Palay, 1974; Caddy & Biscoe, 1976; Rakic & Sidman, 1973b). The cytoarchitecture and synaptic connections are summarised in Figures 1 and 3.

Granule cells. These are the most numerous neurons in the brain (approximately 2.2×10^9 per cerebellum). The granule cells are excitatory and synapse on the other four neuronal types in the cortex through their "T" shaped axons, the parallel fibres. The granule cell layer lies below the level of the Purkinje neurons, and the axons of the granule cells project up toward the cerebellar surface, bifircate and run horizontally in the molecular layer. The molecular layer consists of closely packed parallel fibres and the dendrites of the other four neurons. All granule cell synapses on the inhibitory neurons occur exclusively on dendrites in the molecular layer.

Purkinje neurons. The Purkinje neurons are the largest neurons in the cerebellum (the approximate diameter of the perikaryon is 25 μm) and they appear as a single row of cells between the granule cells and the molecular layer. These cells are much fewer in number than the granule cells (1.2×10^6 per cerebellum). Purkinje cell dendrites extend into the molecular layer where they receive synapses from the parallel fibres. The dendritic tree of the Purkinje cells is flattened and oriented perpendicular to the parallel fibres so that

these neurons are contacted by many parallel fibres. The number of contacts has been estimated at 8×10^4 per Purkinje cell. The axons of the Purkinje neurons leave the cerebellar cortex and form inhibitory synapses with the cerebellar nuclei located in the white matter. These axons are the sole output of the cerebellum. Purkinje axon collaterals also synapse with the other three inhibitory neurons of the cerebellum and other Purkinje neurons.

Golgi neurons. The dendrites of these neurons project up into the molecular layer, where they are excited by the parallel fibres. The dendritic tree is not flattened as in the Purkinje neurons but cylindrical. The Golgi cell axons branch extensively in the granule cell layer where they form inhibitory synapses on the granule cell dendrites. The Golgi axons synapse on granule cell dendrites and form part of the cerebellar glomerulus complex which includes the mossy fibre terminals (see Figure 2). Approximately 10^4 granule cells are inhibited by one Golgi neuron. The Golgi neuron dendrites and soma are thought to be contacted by mossy fibres (Eccles et al. 1967; Hamori & Szentagothai, 1966; Altman, 1972c); however Fox et al. (1967) failed to find such contacts.

Basket and stellate neurons. These cells lie in the lower half of the molecular layer, and there are about 3×10^5 stellate and basket cells per cerebellum. The stellate cells are located more superficially than the basket cells which are located in the lower third of the molecular layer. The dendritic arbor of the basket cells extends up towards the cerebellar surface and is flattened in the same plane as the Purkinje neurons. These inhibitory neurons send out axons, which

project along the plane in which these cells are oriented, and synapse with the dendrites of Purkinje cells some 1-2 cells distant from the area in which the basket cell is located. Descending basket axon collaterals also synapse with the Purkinje cell body. Because of the orientation of the basket cells, Purkinje neurons which are excited by parallel fibres are not inhibited by basket cells receiving the same parallel fibre excitation. Functionally the stellate cells are very similar to basket cells but as their name implies the dendritic tree extends in all directions in the saggital plane of the molecular layer. However, the axons of these neurons do not have collaterals which synapse with the Purkinje cell body.

Afferent fibres. There are two major inputs to the cerebellum; the climbing fibres and the mossy fibres, both of which are excitatory.

Mossy fibres synapse with the granule cells. These fibres branch profusely, often sending branches to several folia, and terminate in the cerebellar glomeruli, which are specialised synapses, consisting of the mossy fibre terminal which contacts the claw like granule cell dendrites and a Golgi axon contact. A single mossy fibre gives rise to 20 or more glomeruli and the dendrites from approximately 20 granule cells may contact each glomerulus. As already discussed, the mossy fibres are also known to contact the Golgi neurons. The information carried in the mossy fibres comes from many areas including, the cerebral cortex (via the pons), muscle receptor organs, the reticular formation and the vestibular system.

Climbing fibres contact Purkinje cell dendrites, each neuron being associated with one fibre which "climbs" along the primary and secondary branches of the Purkinje cell arbour, synapsing at frequent intervals. These fibres arise mainly from the inferior olive which receives afferents from proprioceptors in the cerebral cortex.

There is at least one more afferent fibre system in the cerebellum. Autoradiographic and histochemical studies show that these fibres are noradrenergic (arising in the locus coeruleus) and synapsing with the primary and secondary dendrites (Hokfelt & Fuxe, 1969; Olsen & Fuxe, 1971; Bloom et al. 1971). In addition 5-hydroxytryptamine fibres have been observed in the cerebellum which are thought to originate in the raphe nucleus (Hokfelt & Fuxe, 1969; Dahlstrom & Fuxe, 1965) however the precise location of their synapses is unknown.

Efferent fibres. As already mentioned the sole output of the cerebellar cortex is the Purkinje axons which synapse with the neurons of the deep cerebellar nuclei located in the white matter. These nuclear cells relay information to the spinal cord and sensorimotor cortex.

The source of the afferent fibres and the destination of the efferent system in the cerebellum indicate that this brain region plays a major role in the processing of information concerning

movement and posture. The experimental and clinical evidence supports this idea. The most obvious feature of gross cerebellar dysfunction is ataxia (Dow & Morruzi, 1958). Lesions of the cerebellum also result in dysmetria. Thus movements directed towards a particular object are poorly executed and the subject tends to overshoot and undershoot the target (reviewed by Dow & Morruzi 1958; Ruch, 1960). All the evidence therefore points to the cerebellum modulating motor function. This would inevitably involve the cerebellum acting as a computer, evaluating and acting on information concerning the status of a particular motor function. The repetitive nerve networks lend themselves to the idea of the cerebellum as a computer and as early as 1967 Eccles et al. suggested how the cerebellum might act in such a way. The original idea has been elaborated on and various models have been put forward to account for the computational properties of the cerebellum (Eccles, 1973; Albus, 1971; Marr 1969). However as pointed out by Ito (1979) although the cerebellum (which structurally resembles a computer) may contribute to the functioning of the organism as if it were a computer, the way in which this brain region achieves this effect is still not sufficiently understood.

FIGURE 1 Stereodiagram of the mammalian cerebellum showing the 5 main neuronal types, granule neurons (green), Purkinje neurons (red) and the Golgi (Go), basket (Ba) and stellate neurons (St) shown in black. The two main afferents, the climbing fibres (Cl) and mossy fibres (Mo) are outlined in blue. This diagram was reproduced from Eccles et al. (1967).

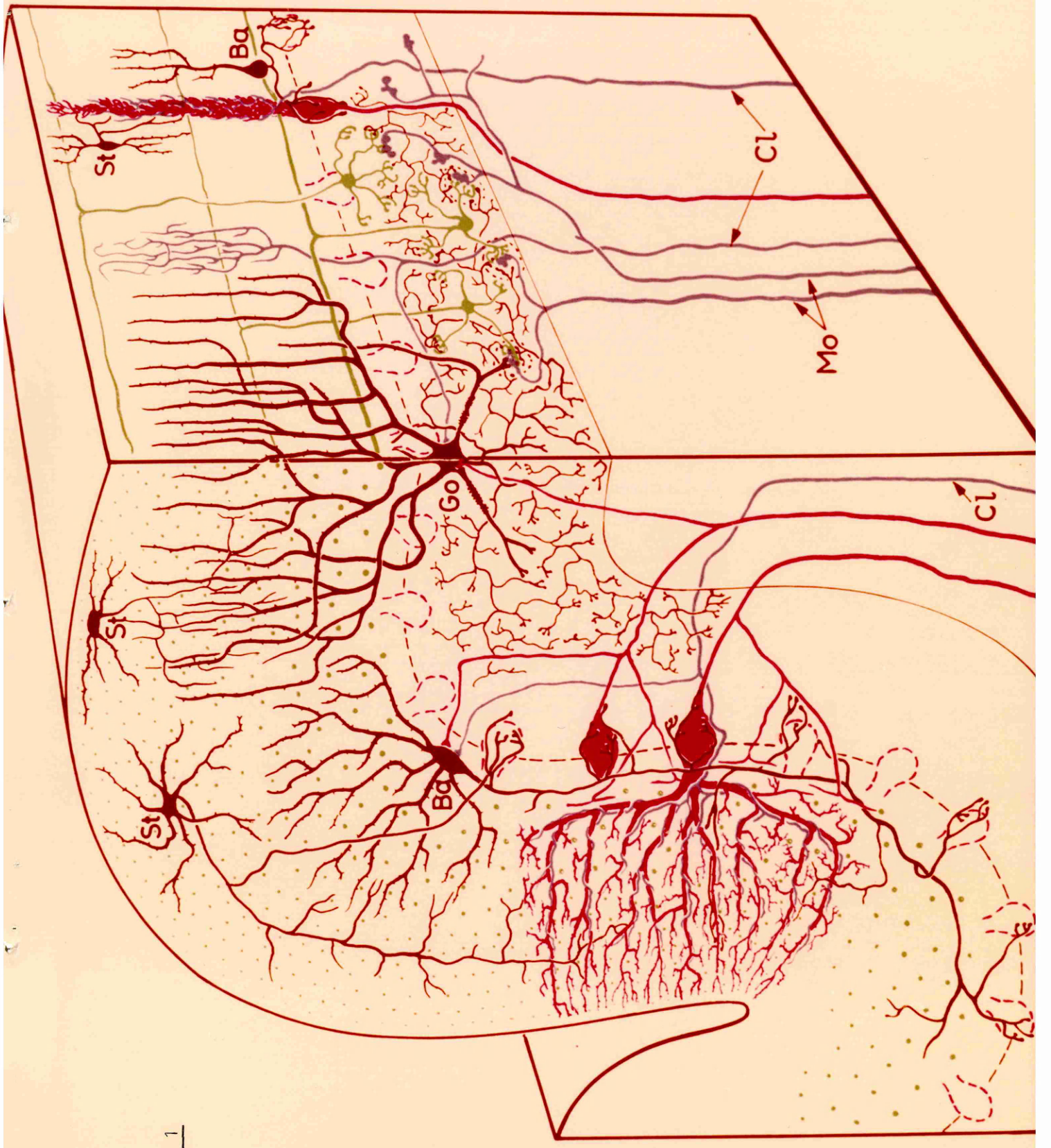
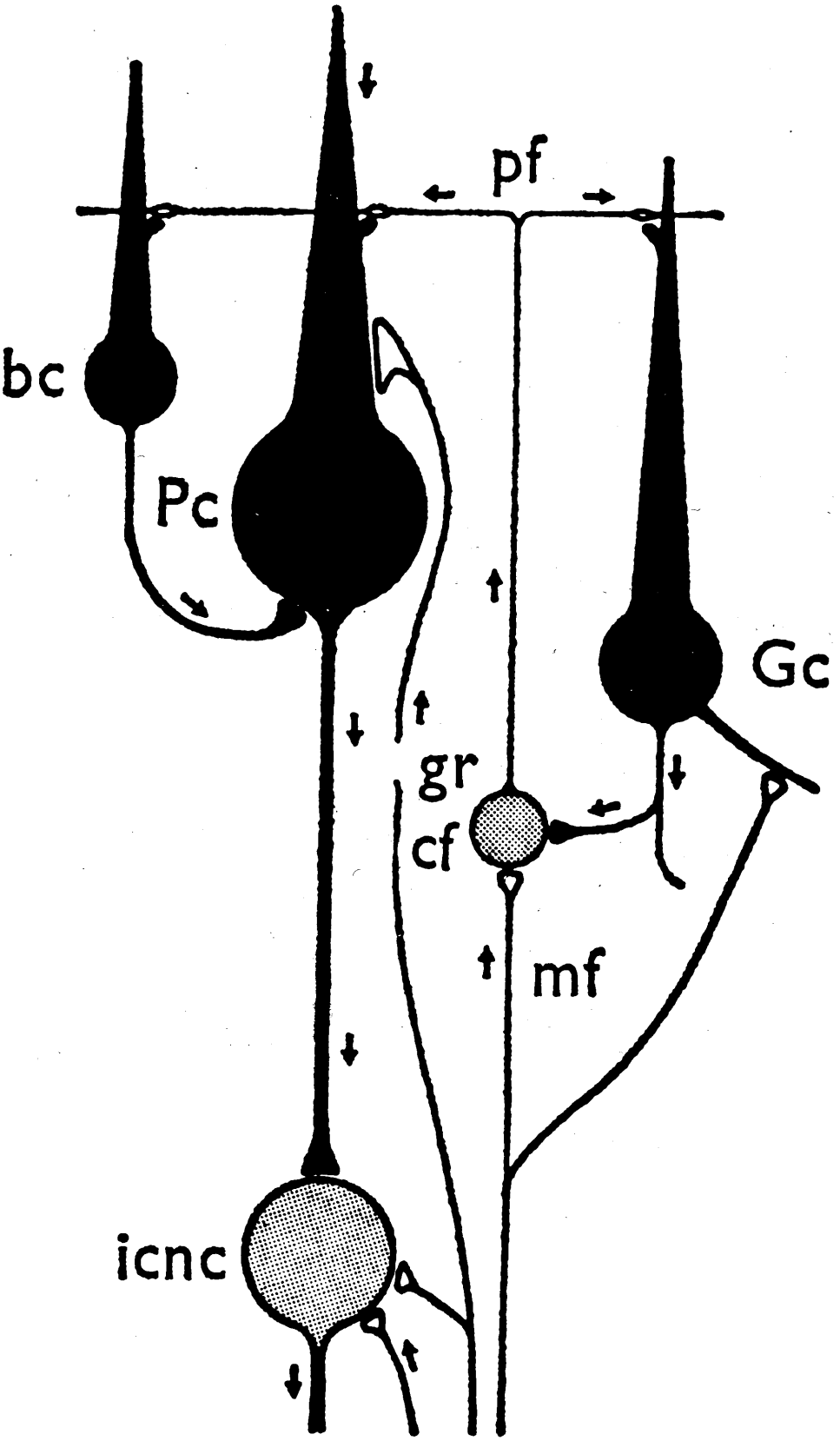


FIGURE 1

FIGURE 2 Simplified wiring diagram of the mammalian cerebellum showing the main nervous connections. The inhibitory cells are shown in black and the arrows indicate the direction of transmission. Pc = Purkinje cell, Gc = Golgi cell, bc = basket cell, icnc = intracerebellar nuclear cell, pf = parallel fibre, cf = climbing fibre, mf = mossy fibre. Reproduced from Eccles (1973).

FIGURE 2



1.2 Cerebellar development

The mouse cerebellum appears as a thickening of the anterior roof of the fourth ventricle around embryonic days 10-12 (Miale & Sidman, 1961). Using classical histogical techniques Ramon y Cajal (1955) observed the migration of Purkinje and granule neurons which feature prominently in the development of this brain region. However autoradiographic techniques, using ^{3}H-thymidine which labels dividing cells, allow the experimenter to determine the exact time and place at which particular cell types arise and subsequently to follow the migration of those cells. Using this technique in the mouse Miale and Sidman (1961) were able to show the time at which each of the five neuronal cell types was formed from its germinal cell and to follow the migration of these cells to their adult positions. The Purkinje and Golgi cells were shown to be formed from the ependymal layer on the roof of the 4th ventricle around embryonic days 11-13 and 12-15, respectively. Following the final mitosis these neuron precursors migrated upwards towards the developing cerebellar cortex. All the other neurons however arose from germinal cells located just below the pia i.e. the external granular layer. The granule cells formed during the late embryonic stages up to 15 days postnatally migrated downwards, past the Purkinje cell layer to the internal granular layer. The autoradiographic study showed that the stellate cells were formed from the external granular layer during the first postnatal week (Miale & Sidman, 1961), however no migrations of these cell types was observed.

Although a number of cell types appear before and just after birth in the mouse, the maturation of the cerebellum occurs mainly postnatally and is essentially complete by 21 days (Meller & Glees, 1969; Larramendi, 1969). The time course for cerebellar maturation in the rat is very similar although a day or two slower (Altman 1972 a, b, c). A brief outline of the development of the cerebellar neurons and their major connections is given below (see also Figure 3 for a summary of the development of some of the major synaptogenic events in the rat cerebellum).

Purkinje neurons. Although formed before birth these cells do not take up their characteristic adult positions, i.e. a single row of neurons, until around 3-4 days after birth in both rat and mouse (Altman, 1972b; Rakic & Sidman, 1973a). At this age the Purkinje neurons have axons which project down into the white matter to the cerebellar nuclei (Larramendi, 1969; Meller & Glees, 1969; Altman, 1972b). The climbing fibres form the first contact with the Purkinje neurons synapsing initially with protrusions from the cell body (perisomatic processes). These climbing fibres contacts are first observed on day 6 and are numerous by day 7 in the mouse (Larramendi, 1969); these events occur a day later in the rat (Altman, 1972b).

Although the climbing fibres make their first contacts with the Purkinje cell body, in the adult they synapse on the primary and secondary Purkinje dendrites. This is achieved by a little understood process referred to by Larramendi (1969) as translocation.

In the mouse this shift in the climbing fibres from the cell body to the primary and secondary dendrites occurs between day 6 and 14. The time course of these events is similar in the rat (Larramendi, 1969; Altman, 1972b). In adults the climbing fibres form a one-to-one association with the Purkinje cells (Ramon y Cajal, 1955; Eccles, et al. 1966). However in immature rats (8-9 days) electrophysiological evidence indicates that most of the Purkinje cells are innervated by two climbing fibres although the normal adult pattern is achieved around 15 days (Crepel et al. 1976b). Thus it would appear that in addition to the migration of climbing fibres, degeneration of some climbing fibre collaterals also takes place during the second postnatal week.

Basket cell axons make contact with the Purkinje cell body in the adult, and these synapses are formed shortly after the climbing fibres are translocated (day 10 in the mouse). These contacts increase in number up to 14 days (Larramendi, 1967a). The first basket axon contacts are observed around day 12 in the rat (Altman, 1972b). The basket and stellate cells also synapse with Purkinje dendrites but these appear to be formed at a later stage. Altman (1972b) has observed stellate cell axon contacts on Purkinje dendrites at 15 days in the rat. Although Larramendi (1969) was unable to say precisely when such synapses were first formed, he estimated it to be after 10 days. The most numerous synapses on the Purkinje neurons are formed by the parallel fibres (Eccles et al. 1967). Since the granule cells are migrating over the period 0-15 days in the mouse (Miale & Sidman, 1961), the period over which the maturation of the parallel fibre/Purkinje contacts occurs might be

expected to be similarly prolonged. In the mouse parallel fibre contacts with Purkinje cells can be seen as early as day 7 or 8 and on day 14 the molecular layer consists of large numbers of mature and maturing parallel fibres (Larramendi, 1969). At this age however, Meller & Glees (1969) came to the conclusion that the Purkinje cells had attained the maximum number of spines. Since the granule cells are still being produced up to 15 days (Miale & Sidman, 1961) it seems more likely that development of parallel fibre synapses proceeds for a few days after day 15. Development of these synapses is much later in the rat cerebellum, the first synapses appearing around 12 days. However Woodward et al. (1971) showed using ethanolic phosphotungstic acid, which specifically stains synapses (Bloom & Aghajanian, 1968), that contacts were made on Purkinje cells around day 3-4, and their electrophysiological studies showed that responses to parallel fibre stimulation could be observed at this age. Despite this anomaly Altman's study (1972b) clearly shows that most of the parallel fibres are laid down between 15 and 21 days, although synaptogenesis is not complete in the upper molecular layer until sometime between 21 and 30 days.

The granule cells. These cells undergo a number of developmental stages. Following the final mitosis, the granule cells orient themselves in the antero-posterior plane (see Figure 1) and the immature parallel fibres grow out from the granule cell. The cell body now located centrally along the maturing parallel fibre then migrates down into the internal granular layer leaving in its path its 'T-shaped' axon. The way in which the granule cells migrate

across the maturing molecular layer is unclear. Rakic (1971) has suggested that this might be achieved by an association between the granule neurons and the radially oriented Bergmann glia, which span the molecular layer. This idea was given some credence by the finding that such glial guides were absent from the cerebella of wv mice, in which the granule cells fail to migrate (Rakic & Sidman, 1973a)although contrary reports have since appeared (see Section 1.3). Bignami & Dahl (1973) reported that Bergmann glia fibres did not become radially oriented until several days after birth in the rat and Das et al. 1974 concluded that the majority of these cells were formed 9 days after birth,i.e. after granule cell migration had started. In addition Altman (1975) examined the alignment and development of Bergmann glia in normal and X-irradiated animals. The parallel fibres of the X-irradiated rats were misaligned as were the Bergmann glia. Altman concluded that the Bergmann glia alignment was a consequence of parallel fibre orientation and that these cells were formed too late in development to influence the migration of the granule neurons. Thus the role of the Bergmann glia in the migration of granule cells is widely disputed.

The parallel fibres synapse (making up the presynaptic element) with all the other neuronal types of the cerebellum. The maturation of the synapses between parallel fibres and the Purkinje cells has been mentioned and to avoid repetition the maturation of other synapses concerned with the parallel fibres will be dealt with here. Larramendi (1969) was unable to say exactly when the first stellate and basket cells were contacted by parallel fibres. However he

observed well differentiated stellate cells with numerous parallel fibre synapses at 14 days. Also Larramendi came to the conclusion that these cells developed their synapses simultaneously at the dendritic and axonal poles. This would mean that basket cells would receive their first parallel fibre synapses around day 10, and basket cells would be contacted some time after 12 days. In the rat, parallel fibre synapses were seen in basket cells on day 7 and similar synapses were observed on differentiating stellate cells at the beginning of the second week (Altman, 1972 a,b). It should be noted that there appear to be differences in the maturation of parallel fibre synapses in rat and mouse. Altman's study (1972 a,b) shows that parallel fibres contact basket cells before they contact the Purkinje neurons and that these basket cells also achieve their synapses on Purkinje neurons before the first parallel fibres. However Larramendi's study (1969) shows that the Purkinje cells were contacted by basket cell axons 2-3 days after the parallel fibres. The only remaining cell type with which the parallel fibres form synapses are the Golgi cells. In the mouse differentiating Golgi cells are seen with a few parallel fibre synapses in 7 day old mice (Larramendi 1969). Golgi cell synapses in the molecular layer were not examined in Altman's study probably due to the difficulty in recognizing Golgi dendrites in electron micrographs (Altman, 1972 a,c). However maturation of the Golgi axons takes place around day 12 and since this is the age at which mature parallel fibres are seen in the molecular layer (Altman, 1972 a), it seems likely that parallel fibre synapses occur on Golgi cells at this time.

In the granule cell layer the granule cells form dendrites which are contacted by mossy fibres and Golgi axons to form the cerebellar glomeruli. The maturation of the glomeruli is a protracted process (Larramendi, 1965, 1967 b). Initially the granule cell dendrites are contacted solely by the mossy fibre rosettes (around 7-10 days in the mouse) and Golgi axons were not seen contacting granule cell dendrites until after the formation of mossy fibre contacts, i.e. after day 7 (Larramendi 1965). The maturation of the glomeruli is similar in the rat with the first contacts on granule cells being observed at the beginning of the third week (Altman, 1972 c). However the electrophysiological data of Puro & Woodward (1977) indicate that such synapses exist as early as 7 days.

Basket and stellate neurons. These interneurons are the only neurons in the cerebellum which do not migrate during development (Miale & Sidman, 1961). After undergoing their last cell division these cells are laid down on the developing molecular layer. The interneurons orient themselves across the parallel fibres and newly formed parallel fibres are laid down over these cells (Altman, 1972 a; Larramendi, 1969; Rakic, 1972). These cells then form synapses with the parallel fibres with which they are in contact. The basket cells are generated first, and since the majority of parallel fibres are formed above the cell body of these cells, the majority of their dendrites grow upward into the maturing molecular layer taking on a basket shape (Rakic, 1972). Stellate cells however are laid down later, in the middle of the molecular layer, and the last stellate cells are formed during the major period of granule cell migration. Thus

a considerable number of parallel fibres are laid down on top of these stellate cells (Miale & Sidman, 1961; Altman, 1972 a; Larramendi, 1969). The stellate cells are therefore able to form synapses with parallel fibres above and below the level of their cell bodies and consequently take on a star shape (Rakic, 1972). The major synaptic contacts formed by and on these cells has already been discussed.

Golgi cells. Like the Purkinje neurons these cells are formed before birth and migrate from the ependymal layer into the cerebellar cortex (Miale & Sidman, 1961). Differentiating Golgi cells have been observed in the mouse as early as 7 days (Larramendi, 1969) and maturing Golgi dendrites and immature axons have been observed in 5-7 day rats, (Altman, 1972 c). Except for the mossy fibres which synapse with the Golgi cell body all the other major contacts on and by Golgi neurons have been discussed. In the rat the mossy fibre synapses are seen in animals of 15 days or older (Altman, 1972 a). Both Larramendi (1969) and Meller & Glees (1969) failed to definitely identify mossy fibre synapses on the Golgi cell body although Larramendi (1969) did report axosomatic synapses on Golgi neurons in 7 day old mice.

It should be noted that in this section most of the information given is for the first contacts seen for particular neuronal elements during development and that some of these contacts are not seen in any number until several days later. Figure 3 shows the ages in the rat at which particular cell contacts become prominent and there is some discrepancy between this and the age at which these contacts first occur as described in the text.

FIGURE 3 Diagramatic representation of some of the major events occurring during the maturation of the rat cerebellum and outlined in Section 1.2. Reproduced from Altman (1973b).

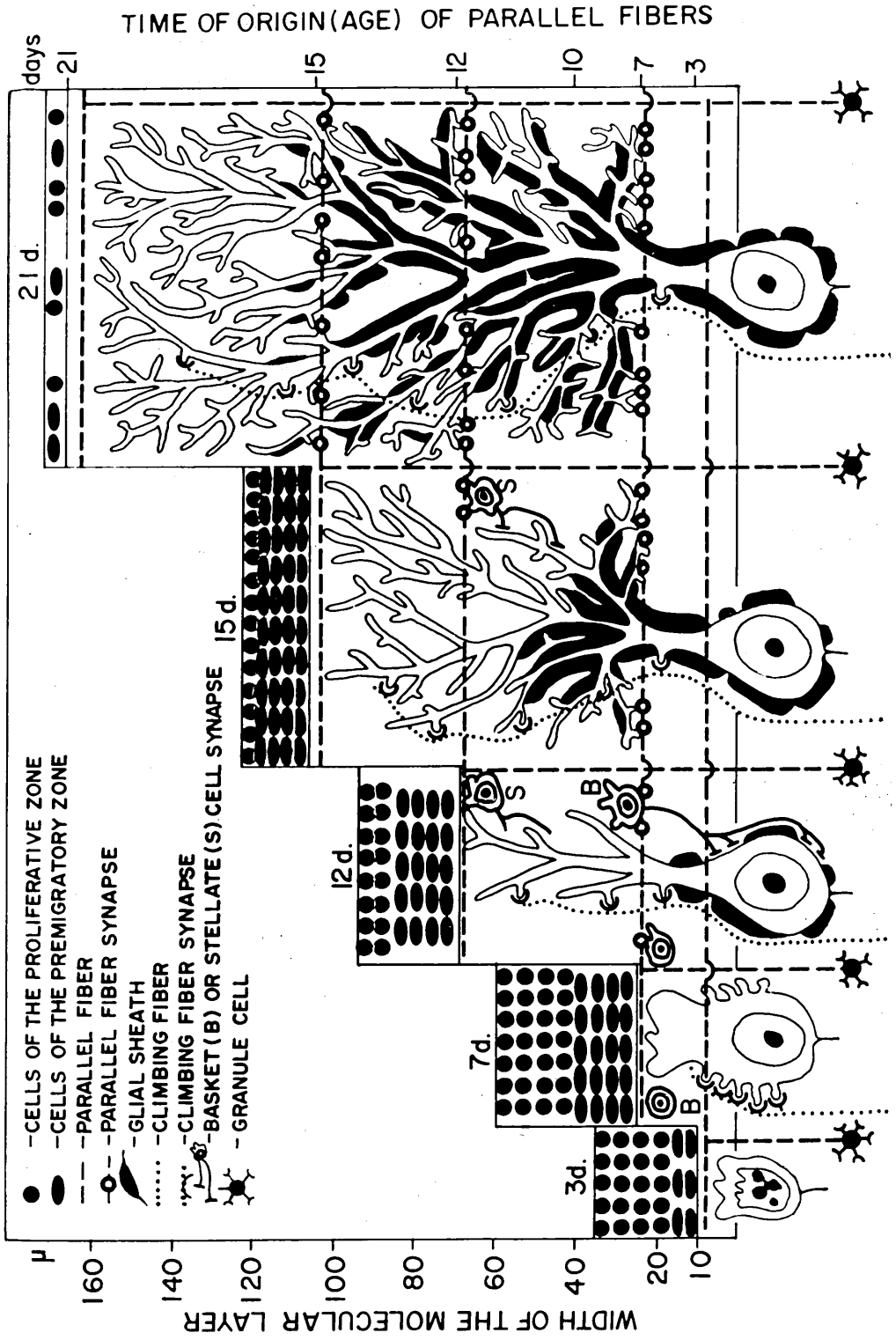

TIME OF ORIGIN (AGE) OF PARALLEL FIBERS
days
21
15
12
10
7
3
21 d.
15 d.
12 d.
7d.
3d.
S
B
B
WIDTH OF THE MOLECULAR LAYER
μ
160
140
120
100
80
60
40
20
10
-CELLS OF THE PROLIFERATIVE ZONE
-CELLS OF THE PREMIGRATORY ZONE
-PARALLEL FIBER
-PARALLEL FIBER SYNAPSE
-GLIAL SHEATH
-CLIMBING FIBER
-CLIMBING FIBER SYNAPSE
-BASKET(B) OR STELLATE(S) CELL SYNAPSE
-GRANULE CELL

FIGURE 3

1.3 Neurological mutants

Over 100 neurological mutants have been identified in the mouse, most of which have been catalogued by Sidman et al. (1965). These mutations affect a wide range of neurological structures, which include, cerebellar and cerebral malformations, disorders of the neural crest, ear and retina. Also there are many disorders which are not attributable to any particular region but are presumably the result of more general pathological changes in the nervous system. In the following study, four mutants have been examined. These mutants are weaver (wv) reeler (rl), staggerer (sg), in which the development and organisation of the cerebellar cortex is severely disrupted (Sidman, 1968), and jimpy (jp). The neuropathology of jimpy is quite different from that of the other three. The neuronal organisation of the jp mutant is normal but myelination of axons in the central nervous system of these animals is markedly reduced (Sidman et al. 1964; Meier et al. 1974). As will be discussed later in this section, marked abnormalities are also apparent in the cytoarchitecture of the rl cerebral cortex. No such features have been reported for wv or sg. However Lane et al. (1977) have reported significant differences in the levels of monoamine neurotransmitters in the wv cerebral cortex. Whether this is a direct result of the mutation or a consequence of impaired cerebella function (i.e., a secondary effect of the mutation) is unclear.

A more detailed outline of the neuropathology of these mutants is given below. The gene symbols (wv, rl, etc.) are used to denote animals which are affected by those mutations.

Weaver. This autosomal recessive mutant was first described by Lane (1964). The mutation is characterised by instability of gait, hypotonia, tremor and poor survival (Sidman et al. 1964). The cerebellum of the homozygous wv is much smaller than that of the wild type (Sidman, 1968).

The heterozygote cerebellum is also slightly smaller than control (Rezai & Yoon, 1972; Rakic & Sidman, 1973a) although heterozygotes are behaviourally normal.

Histological studies show that the most striking feature of "adult" wv cerebellar cortex is the almost total absence of granule cells (Sidman, 1968; Rezai & Yoon, 1972; Rakic & Sidman, 1973 a,b; Hirano & Dembitzer, 1973) although some granule neurons survive particularly in the ventrolateral areas of the cerebellar hemispheres (Sidman, 1968). During the development of the wv cerebellum the granule cells are formed at the normal time, but the rate of migration of these cells through the molecular layer is markedly reduced, and most of these cells die in the external granular layer, having failed to migrate (Sidman, 1968; Rakic & Sidman, 1973a; Rezai & Yoon, 1972). The deficit in granule cell migration and loss of granule cells also extends to heterozygotes although the deficit is not as marked as in wv (Rezai & Yoon, 1972; Rakic & Sidman, 1973a). Rakic & Sidman (1973a,c) have examined more closely the failure of granule cells to migrate in wv. As already discussed in Section 1.2 it has been suggested that migration in the cerebellar cortex takes place in association with radially oriented guides - the Bergmann glia (Rakic, 1971). During the development of the wv mutant and to a lesser extent in the heterozygotes, Rakic & Sidman (1973 a,c) reported that the Bergmann glia were reduced in number and only a few displayed normal cytological features. Sidman & Rakic (1973a) came to the conclusion that the granule cells were probably not the primary target of the weaver locus since the granule cell precursors appear normal in mutants. In addition, granule cell death only becomes significantly elevated above control soon after migration begins in controls and granule cells which do migrate, survive and appear normal. On the basis of these findings and the evidence

concerning the Bergmann glia Sidman & Rakic (1973 a,c) postulated that granule cell death occurred as a consequence of the failure of these cells to migrate; which was the direct result of Bergmann glia abnormalities. Furthermore they suggested that the Bergmann glia were probably the primary target of the weaver locus. However, using the immunological glial marker anti-glial fibrillary acid protein (Bignami & Dahl, 1973, 1974 a), Bignami & Dahl (1974 b) examined the Bergmann glia of wv mutants. They found that in 4 day mutants the Bergmann glia were identical to those in controls and even at 30 days the Bergmann glia of mutants appeared relatively normal, and they were oriented radially from the Purkinje cell layer up to the pial surface. Sotelo & Changeux (1974) essentially confirmed these results in 20 day wv. (See also Section 1.2 for evidence concerning the role of Bergmann glia in granule cell migration). It is not clear why the studies of Sidman & Rakic (1972 a,c) should contrast with Bignami & Dahl (1974) and Sotelo & Changeux (1974 a), although it has been suggested that different genetic backgrounds of the mice studied may be responsible. However, the above findings cast doubt on the theory that the Bergmann glia are the primary target of the mutation. Although Sidman & Rakic (1973a) maintain that granule cells which migrate, survive and appear normal, Sotelo & Changeux (1974a) report that even migrating granule cells die in the more severely affected areas of the cerebellum. Sotelo & Changeux (1974a) thus concluded that the genetic abnormalities were probably associated with the granule cells. However, granule cells cultured from wv cerebellum survive as long as those from normal littermates (Messer & Smith, 1977) indicating that granule cell death is the result of factors external to these cells.

Whatever the site of action of the weaver locus, the granule cell deficit has a marked effect on the organisation of the cerebellum (Sidman, 1968; Rakic & Sidman, 1973b; Hirano & Dembitzer 1973, 1974; Sotelo, 1975 a,b). It seems likely that the abnormalities seen in the "mature" wv cerebellum are the result of granule cell loss and not a more direct effect of the mutation for the following reason. Cerebellar organisation in wv closely resembles that observed in experimentally induced agranular cerebella, where the dividing granule cell precursors are destroyed by, viral infection, X-irradiation or chemical poisoning (Herndon, et al. 1971; Altman & Anderson, 1972, Hirano, et al. 1972; Llinas et al. 1973; Rakic & Sidman, 1973b; Sotelo 1975b). However such treatment also destroys the interneuron precursors and these animals are severely depleted of basket and stellate cells (Altman & Anderson, 1972; Woodward, et al. 1974).

Since all the neuronal elements of the cerebellum are contacted by granule cell axons (Eccles et al. 1967), it is not surprising that all cells in the cerebellum are affected by granule cell loss in wv. The major changes in cerebellar organisation are briefly outlined below.

Although the Purkinje cell bodies are distributed several rows deep instead of forming a single layer, these cells appear superficially normal using classical histological techniques (Sidman, 1968). However these neurons have a reduced dendritic arbor and form few tertiary branches (Bradley & Berry, 1978; Rakic & Sidman 1973 b; Sotelo, 1975 a).

Spiney branchlets, which normally form synapses with parallel fibres are absent from wv Purkinje neurons (Sotelo, 1975 a,b; Rakic & Sidman, 1973. Hirano & Dembitzer, 1973). However, the primary and secondary dendrites are studded with spines, which closely resemble those contacting parallel fibres in the normal cerebellum. These spines show the characteristic membrane thickening, similar to normal post-synaptic elements and extracellular material, such as is seen in synaptic clefts is also associated with these processes. Neuronal contacts are rarely observed on these spines and most of them are enveloped by glia (Rakic & Sidman, 1973b; Hirano & Dembitzer, 1973; Sotelo, 1975 a,b). Interneuron and climbing fibre input to the Purkinje neurons is also altered. Basket fibres do not form the classical synapses with the Purkinje cell body, and although contacts are formed by the interneurons on the Purkinje cell body and dendrites they are reduced in number. The surface density of climbing fibre varicosities on Purkinje neurons is increased (Sotelo, 1975 b). Electrophysiological studies indicate that as in developing animals, wv Purkinje cells are multiply innervated by climbing fibres, in contrast to the one-to-one relationship maintained between these neuronal elements seen in normal cerebellar cortex (Crepel et al. 1976 b; Crepel & Mariani, 1976; Puro & Woodward, 1977; Eccles et al. 1967). Multiple innervation of Purkinje cells by climbing fibres is also seen in animals depleted of granule cells by X-irradiation (Crepel et al. 1976 a; Woodward et al. 1974).

The basket and stellate cells are generated at the normal time during development but, in contrast to the Purkinje cells their dendrites are stunted and randomly oriented (Rakic & Sidman, 1973 b;

Sotelo 1975b). However like the Purkinje cells these interneurons develop post-synaptic specialisations in the absence of the granule neurons (Sotelo, 1975 b).

There is little information concerning the Golgi neurons in the cerebellum of the wv mutant. However the migration of these neurons into the cerebellar cortex appears normal, although the final position of the Golgi neurons is altered, possibly due to the smaller size of the mutant cerebellum, as already discussed for the Purkinje neurons (Rakic & Sidman, 1973 a,b).

In the absence of granule cells the mossy fibres form more than the usual number of contacts with Golgi neurons. Some aberrant granule cells are found in the "molecular layer" and mossy fibres are found in contact with these cells (Rakic & Sidman, 1973 b; Sotelo, 1975 b).

Sotelo (1975 b) has reported the presence of heterologous synapses i.e. synapses between neurons which do not usually contact one another in the normal cerebellum. Most of these contacts occur on Purkinje cell spines normally contacted by parallel fibres and heterologous contacts include mossy fibre rosettes and granule cell bodies/ dendrites. Similar abnormal synapses have been observed in experimentally induced agranular cerebella (Altman & Anderson, 1972; Llinas et al. 1973). The importance of this synaptic remodelling is unclear since most of the Purkinje cell spines remain unoccupied (Sotelo, 1975 b) and according to Rakic (1976) such abnormal synapses also occur occasionally in the normal cerebellum.

In summary the evidence suggests that synaptic reorganisation involving neuronal elements which do not normally form contacts is not a major phenomenon, in contrast to certain other brain areas, where lesions result in the establishment of abnormal new contacts (Raisman, 1969; Lynch et al. 1973; Schneider, 1970). However some synaptic remodelling of normal synapses does take place, i.e. multiple innervation of Purkinje neurons by climbing fibres and increases in the number of synaptic contacts made on Golgi cells by mossy fibres (Crepel et al. 1976 a; Rakic & Sidman, 1973 b).

Staggerer. This agranular mutant was first described by Sidman et al. (1962) and like the wv this mutation is autosomal and recessive. Behaviourally sg resembles wv, and the cerebellum of this mutant is much smaller than that of the wild type; even smaller than the wv cerebellum (Sidman, 1968).

Unlike the wv the granule cells of sg migrate into the external granular layer, albeit prematurely, (Yoon, 1972) and these cells develop and superficially resemble mature granule cells. The parallel fibres of these cells make synaptic contact with all their normal targets with the exception of the Purkinje cells and granule cell dendrites receive their normal synaptic inputs in the glomeruli (Sidman, 1972; Sotelo & Changeux, 1974 b). The Purkinje cells of this mutant are also strikingly abnormal and under the light microscope these neurons appear immature and stunted (Sidman, 1968). Electron-microscopic studies show that in the developing mutant these neurons also lack the dendritic spines, contacted by parallel fibres in normal animals, and which are also found in the wv mutant despite the

absence of the fibres (Sotelo, 1973; Sotelo & Changeux, 1974 b; Sotelo, 1975 a; Hirano & Dembitzer, 1975; Landis & Sidman, 1978). However a few spines have been observed in older animals (20-28 days) (Sotelo, 1975a; Hirano & Dembitzer, 1975). This led Hirano & Dembitzer (1975) to propose that Purkinje cell spine production is late and abortive in the sg mutant. Evidence that granule cells are able to recognize the Purkinje neurons in sg comes from the finding that the immature nonsynaptic segment of parallel fibres form adhesion zones on the smooth surface of the Purkinje dendrites. In normal animals such contacts are taken to be the initiation of synapse formation between these neuronal elements (Sotelo & Changeux 1974 b). Although these adhesive zones do not resemble normal synapses, Crepel & Mariani (1975) have reported that such undeveloped synapses may function, albeit inefficiently. Because of the abnormality of the Purkinje cells, and the relatively normal, although short-lived, development of the granule neurons, it has been suggested that the Purkinje neurons are the primary target of the sg locus (Sidman, 1972; Landis, 1971; Landis & Sidman, 1978). Sotelo & Changeux (1974 b) have further proposed that granule cell death may be a direct consequence of these cells being unable to synapse with aberrant Purkinje neurons. Further evidence that the sg locus directly affects the Purkinje neurons comes from chimera studies. Chimeras are animals produced by the combination of two separate embryos. In order to obtain sg/normal chimeras an eight cell embryo from a normal mouse is combined with an eight cell sg embryo. The resulting blastocyst is then returned to the uterus. The cells of animals thus formed are made up of cells of two types, i.e. sg and normal cells. The cells of normal parents can be distinguished from those of mutant producing

parents in the chimera because sg cells also possess high levels of the enzyme β-glucuronidase, which can be identified histochemically (see Mullen, 1977 for more detailed description of methods). Staggerer Purkinje neurons in such chimeras are aberrant despite the presence of adjacent normal Purkinje and granule cells, strongly suggesting that the cause of the Purkinje cell malformation is intrinsic to these cells (Herrup & Mullen, 1976; Mullen, 1977). Yoon (1972) studied autoradiographically the proliferation and migration of the cells of the external granular layer using ^{3}H-thymidine (see Section 1.2). He was able to show that there was a reduction in the proliferation of these cells and that they migrated prematurely from the external granular layer. On the basis of this information, Yoon (1972, 1976) proposed that the sg mutation affected the granule cell population directly, in addition to the Purkinje neurons. However Landis & Sidman (1974, 1978) suggest that the Purkinje neurons may modulate granule cell proliferation, a view also held by Sotelo & Changeux (1974 b). They further postulate that in sg this modulation is altered resulting in granule cell hypoplasia (Landis & Sidman, 1974, 1978). Recently Herrup & Mullen (1979) performed a quantitative study of large neurons in 30 day sg. They concluded that depending on the number of Golgi cells, which are difficult to distinguish from Purkinje neurons in sg, the deficit of Purkinje neurons in sg ranged from 60 - 90 percent. Thus it seems that the effect of the sg locus on the Purkinje cell population is more severe than was hitherto realised.

Although the synaptic organisation of the sg cerebellum has been less studied than in wv a number of reports have been concerned with this subject. Degeneration of parallel fibres begins around day 7

reaching a maximum between day 16 and 21 (Landis & Sidman, 1978). These observations have been essentially confirmed by Sotelo & Changeux (1974b) and Hirano & Dembitzer (1975). Death of the granule cells results in the loss of the postsynaptic elements in the cerebellar glomeruli (Landis & Sidman, 1978) and according to Sotelo and Changeux (1974 b) this also brings about degeneration of the mossy fibres. Despite the loss of the parallel fibres the stellate and basket cells appear normal although their dendritic arbor may be reduced (Landis & Sidman, 1978). The Purkinje neurons are contacted by climbing fibres on schedule, and although these synapses survive into adulthood they remain of the immature type, i.e. fibres synapsing with processes on the cell soma or proximal dendrites (Landis & Sidman, 1978). However electrophysiological studies indicate that only 40% of Purkinje cells are innervated by climbing fibres (Crepel & Mariani, 1975). These studies also suggest that, while parallel fibres do not form mature synaptic contacts, the initial adhesive zones between these two cells are capable of effecting neurotransmission (albeit inefficiently). Although as mentioned in Section 1.2, it is difficult to differentiate between Golgi and Purkinje dendrites by electron microscopy, Landis & Sidman (1978) identified a number of Golgi cells with extensive dendritic arbors and multiple axons in 16 day old sg. In addition mossy fibre axons have been observed synapsing on the Golgi cell bodies. Despite the death of the granule cells the Golgi axon synapses in the glomeruli survive to adulthood, and there is no evidence to suggest that these inhibitory neurons (and the stellate and basket cells) degenerate during or after the granule cell loss (Landis & Sidman, 1978).

Thus it appears that with the exception of the Purkinje and granule neurons most of the other neuronal elements develop relatively normally. However the large Purkinje cell deficit and the ultimate death of the granule cell population results in a cerebellum smaller than that of wv.

Reeler. This cerebellar mutant (first described by Falconer, 1951) behaviourally resembles the other two cerebellar mutants, and examination of the cerebellar cortex reveals a significant although much reduced population of granule cells (Sidman, 1968). In addition, this mutation also affects the hippocampal formation and the cerebral cortex (Meier & Hoag, 1962; Hamburgh, 1960, 1963; Caviness & Sidman, 1972, 1973 a,b; Caviness, 1973; Devor, et al. 1975). Apart from the loss of granule cells, the most obvious feature of the rl cerebellum is the unusual position of the granule cells relative to the Purkinje cells. The majority of the granule cells occupy what would normally be the molecular layer. Where the normal spatial relationship between granule and Purkinje neurons is attained, the dendritic arbor of the Purkinje cell is normal and reaches up into the shallow molecular layer. However in the areas where these cells are abnormally positioned the arbor of the Purkinje neurons is malformed (Sidman, 1968; Rakic & Sidman, 1972; Rakic, 1976; Mariani et al. 1977). Although the Purkinje neurons are generated on schedule (Sidman 1968) many Purkinje cells lie in the white matter, presumably having failed to reach the cortex. It has been suggested that, since these neurons must migrate through numerous axons in the foetal brain to reach their final position, the Purkinje neurons must be guided to their final position.

Sidman (1968) has postulated that such a mechanism might be at fault in the rl mutant. A similar fault might also explain the failure of the granule neurons (which are also generated at the normal time) to migrate inward past the layer of Purkinje neurons in rl mutants. The idea of a defect in the control of migration is supported by the finding that malformations in the rl brain occur in regions in which cells must migrate through other neurons in order to attain their correct positions, i.e. the cerebral cortex and hippocampus (Angevine & Sidman, 1961; Angevine, 1965; Berry & Rogers, 1965; Sidman, 1968). Experiments with rl/normal chimeric mice indicate that the fault which leads to the abnormal positioning of cells in the cerebellar cortex is extrinsic to the Purkinje and granule neurons since both normal and mutant cells are malpositioned in such chimeras (Mullen, 1977).

As already discussed the abnormality of the cerebellar cortex depends on the relative positions of granule and Purkinje neurons. Up to 40 percent of the Purkinje dendrites occur in areas of the cortex where they are in contact with the narrow molecular layer and the development of cells in such areas is relatively normal (Rakic & Sidman, 1972; Rakic 1976; Mariani, et al. 1977). The synaptic organisation in the areas of the remaining malpositioned Purkinje neurons is as follows. Those Purkinje neurons whose dendrites ramify the granular layer have a disoriented and reduced dendritic arbor. As in wv (Sotelo, 1975 a,b) the dendrites of these Purkinje neurons lack spiney branchlets although the primary and secondary branches of these cells have numerous spines on their surface (Rakic & Sidman, 1972; Mariani et al. 1977). Occasionally parallel

fibres are observed synapsing with spiney branchlets on these cells. Most of the normal contacts on Purkinje cells are maintained in this region and basket cell axons can be seen descending into the granular layer to synapse with the Purkinje cell bodies(Rakic & Sidman, 1972; Mariani et al.1977). Climbing fibres appear to form more than the usual number of contacts with the Purkinje dendrites in this area of the cortex. Electrophysiological studies indicate that these cells are multiply innervated by climbing fibres (Mariani, 1977). In this respect climbing fibre innervation of these Purkinje neurons is similar to that observed in immature and wv cerebellar cortex (Crepel et al. 1976b;Crepel & Mariani, 1976; Puro & Woodward, 1977) and contrasts with one-to-one relationship maintained between these elements in normal and more superficially placed Purkinje neurons (Eccles et al. 1967; Mariani et al. 1977). In addition Mariani et al. (1977) have observed heterologous synapse formation between mossy fibre rosettes and Purkinje dendritic spines. The electrophysiological function of these synapses was also demonstrated in the same study. As already discussed the relevance of such heterologous synapses is unclear since the majority of spines remain asynaptic and heterologous synapses are also occasionally observed in normal animals (Rakic, 1976). The Purkinje cells in the white matter resemble wv Purkinje neurons and receive no inputs from the cerebellar cortex. However, these neurons appear to be multiply innervated by climbing fibres. Although a few heterologous synapses between mossy fibres and Purkinje dendrites have been observed in this region most of the mossy fibres pass through this area to the cortex. Synapses are also formed on the Purkinje cell dendrites

and somas by Purkinje axon collaterals and the axons of interneurons (of unknown origin) also found in the white matter (Mariani et al. 1977).

The cerebellum of the rl mutant is very similar in synaptic organisation to the wv mutant, although in the reeler significant numbers of granule cells are able to establish normal contacts and survive to adulthood. Thus the cerebellum of rl may be considered to be not as disrupted as that of the wv. In contrast, the eventual loss of the granule neurons and the marked deficit of Purkinje neurons indicates that the sg mutation most severely affects the neuronal population of the cerebellum.

Jimpy. This mutant was first described by Phillips (1954). The gene is carried on the X-chromosome and expressed in males carrying the gene. Jp animals can be recognised behaviourally at around 10 days when they show a marked tremor. Older animals exhibit occasional tonic seizures and rarely live longer than one month (Sidman et al. 1965).

The most striking feature of jp animals is the paucity of myelin in the brain although myelination of the peripheral nervous system appears normal (Sidman et al. 1964; Meier et al. 1974; Hirano et al. 1969). The cause of this abnormality is unclear. It appears to be the result of a failure to produce myelin and not the result of demyelination (Meier et al. 1974). However biochemical studies have failed to show any consistent abnormalities in the synthesis of myelin in these animals (Meier et al. 1974; Matthieu et al. 1974; Mandel et al. 1972). A number of studies have been reported showing abnormalities associated with

the oligodendrocytes (which are known to be responsible for the myelination of axons in the central nervous system (Peters et al. 1970) and it has been suggested that the primary genetic defect might be associated with these cells. However Skoff (1976) reported that the abnormality is preceded by astrocyte proliferation and he suggested that in excess these cells might interfere with the interactions occurring between axons and oligodendroglia.

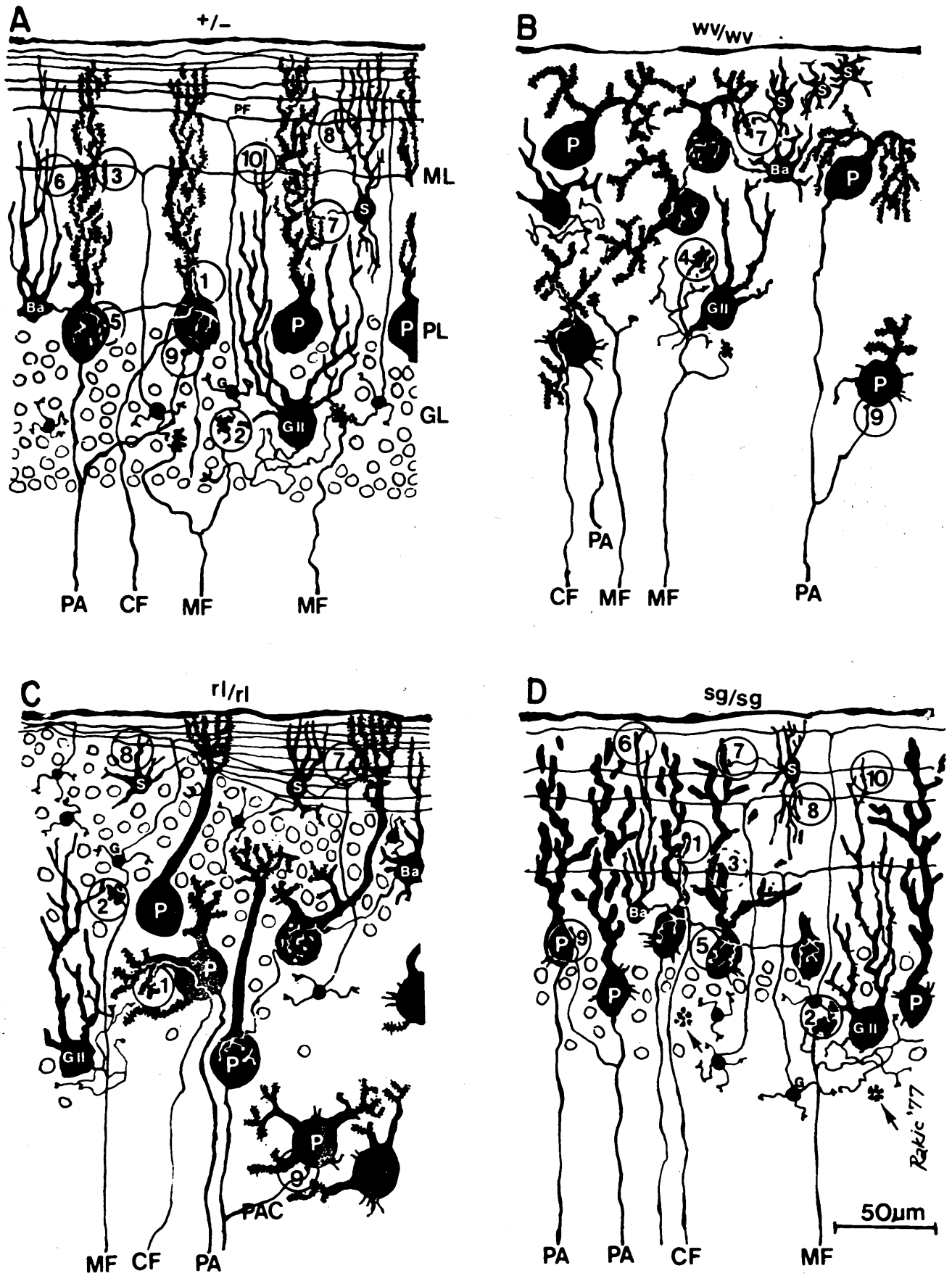
There are, to my knowledge, no reports in the literature of any abnormalities of cerebellar neurons occurring in jp animals, although defects in the growth of myelinated axons in the optic nerve of these animals has been demonstrated (Webster et al. 1976). Recently Kristt & Butler (1978) found reduced dendritic arborization of cerebral cortex stellate neurons and they suggested that this might be in response to reduced neuronal input due to impaired axonal growth. Alterations to cerebellar neurons might also occur in jp if the growth of myelinated axons, which include the mossy and climbing fibres was impaired (Palay & Chan-Palay, 1974). Sotelo et al. (1975) for example has reported an increase in Purkinje spine formation following the destruction of climbing fibres in adult rats.

Detailed aims of the studies involving the use of the neurological mutants are outlined in Chapter 3.

FIGURE 4 Diagramatic representation of neuronal organisation of the cerebellar cortex of normal and weaver, reeler and staggerer, mutant mice.

Key: Ba, Basket cell; CF, climbing fibre; G, granule cell; GII, Golgi cell; MF, mossy fibre; P, Purkinje cell; PA, Purkinje cell axon; PF, parallel fibre; S, stellate cell. The major classes of synapses are circled and numbered: 1, climbing fibre Purkinje cell dendrite; 2, mossy fibre- granule cell dendrite; 3, parallel fibre- Purkinje cell dendrite; 4, mossy fibre- Golgi cell; 5, basket cell axon- Purkinje cell soma; 6, parallel fibre- basket cell dendrite; 7, stellate cell axon- Purkinje cell dendrite; 8, parallel fibre- stellate cell dendrite; 9, Purkinje cell axon collateral- Purkinje cell soma; 10, parallel fibre- Golgi cell dendrite. (Reproduced from Rakic, 1979).

FIGURE 4


A
+/-
PF
ML
PL
GL
Ba
P
GII
PA
CF
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MF
B
wv/wv
S
Ba
P
GII
PA
CF
MF
MF
PA
C
rl/rl
S
Ba
G
P
GII
PAC
MF
CF
PA
D
sg/sg
S
Ba
P
GII
G
Rakic '77
50µm
PA
PA
CF
MF

1.4 Neurotransmitters of the cerebellum

This topic has been reviewed by Tebecis (1974) (see also the more general reviews Curtis & Johnston, 1974; Krnjevic, 1974; De Feudis, 1975). In this section a brief outline of the major postulated neurotransmitters of the cerebellar neurons and afferent fibres is presented.

With the exception of the granule neurons all the neuronal types of the cerebellum are inhibitory (Eccles et al. 1967). All the available evidence, neurophysiological, neuropharmacological and biochemical, indicates that Purkinje, stellate, basket and Golgi neurons use γ-aminobutyric acid (GABA) as their neurotransmitter. The evidence is as follows. The inhibitory influences of these neurons is mimicked by iontophoretically applied GABA (Obata et al. 1970; Bruggencate & Engberg, 1971; Kawamura & Provini, 1970). Furthermore the GABA antagonist bicuculline blocks the inhibitory actions of these neurons (Curtis et al. 1970a; Curtis et al. 1970b; Bisti et al. 1971; reviewed by Curtis, 1979). Immunohistochemical studies show that the enzyme responsible for GABA synthesis, glutamate decarboxylase is localised in the inhibitory neurons (Saito et al. 1974; reviewed by Roberts, 1979). Autoradiographic analysis of (^{3}H) GABA transport reveals that of the cerebellar neurons only the inhibitory cells possess high affinity transport sites for GABA (Schon & Iversen, 1972; Hökfelt & Ljungdahl, 1972; see also section 1.6). Recently a method has been devised for the electron - and light - microscopic autoradiographic visualisation of GABA receptors using the GABA mimetic (^{3}H) muscimol (Chan-Palay, 1978; see also section 1.7). Essentially the study of Chan-Palay

(1978) confirms the role of GABA in the neurotransmitter function of the interneurons and Purkinje cells, with these cells being heavily labelled. However relatively little (^{3}H) muscimol binding was localised on the granule cells, although Chan-Palay (1978) makes no mention of labelling of the cerebellar glomeruli, which is where the presumed GABAergic Golgi cells contact the granule neurons (see section 1.1).

The cerebellum contains relatively large amounts of the inhibitory neurotransmitter candidate taurine (Curtis & Johnston, 1974) and the finding that animals depleted of superficial stellate cells by X-irradiation are also deficient in taurine has been taken to indicate that taurine might function as a neurotransmitter of these inhibitory neurons (McBride et al. 1976b). However as already discussed GABA seems the most likely neurotransmitter candidate of these neurons and direct evidence for the role of taurine is lacking.

The neurotransmitter of the granule cells is unknown but of the putative excitatory neurotransmitters, glutamate appears the most likely candidate. The granule cells excite all other neurons of the cerebellum through their parallel fibres, and iontophoretically applied glutamate excites all cerebellar neurons McCance & Phillis, 1968; Kawamura & Provini, 1970; Chujo et al. 1975). Other evidence for glutamate being a neurotransmitter comes mainly from neurochemical studies on agranular cerebella. The cerebellar cortex contains relatively high levels of glutamate (Johnson & Aprison, 1971), the highest levels being found in the molecular layer (Nadi et al. 1977b;

Berger et al. 1977). However in the agranular cerebella of mutant mice (wv, sg and rl) and X-irradiated or virus treated animals the level of glutamate is markedly reduced while no corresponding change is seen for any other amino acids (McBride et al. 1976a,b; Hudson et al. 1976; Valcana et al. 1972; Young et al. 1974).

In addition Young et al. (1974) reported a marked decrease in the high affinity transport of (^{3}H) glutamate in the agranular cerebella of virally treated animals. Similarly Rhode et al. (1979) reported that in synaptosomes prepared from the agranular cerebella of X-irradiated rats, both the levels of glutamate and uptake of (^{3}H) glutamate were markedly decreased. However in at least one study (Patel et al. 1975) there was failure to find a reduction of glutamate levels in the agranular cerebella of X-irradiated rats. Rea & McBride (1978) showed that glutamate reduction was confined to the agranular cortex and normal levels were found in the white matter and deep cerebellar nuclei. One would predict this if granule cell loss were responsible for the glutamate deficit. However Roffler-Tarlov & Sidman (1978) reported reduced glutamate levels in both the cerebellar cortex and deep cerebellar nuclei of sg and rl mutant mice. The glutamate deficit in the deep cerebellar nuclei cannot be explained by the absence of granule cells. Thus these authors concluded that glutamate reduction in the cerebellar cortex was not necessarily the result of granule cell loss and that this phenomenon was not sufficient evidence for suggesting that glutamate was the granule cell neurotransmitter. A slightly different approach was adopted by Tran & Snyder (1979) to discover the neurotransmitter of the granule cells. Instead of removing the granule cells these authors used the neurotoxic agent kainic acid, which destroys the inhibitory

neurons of the cerebellum but spares the granule cells (Herndon & Coyle, 1977). In kainic acid treated animals glutamate levels were not significantly different from controls although (^{3}H) glutamate transport was reduced by 25-30 percent. However (^{3}H) GABA transport was reduced by 65-70 percent and GABA levels were reduced by 50 percent. Thus the results of Tran & Snyder (1979) were consistent with the idea that granule cells use glutamate as their neurotransmitter, while the remaining neurons use GABA. However the only direct evidence for glutamate functioning asthe granule cell neurotransmitter comes from the study of Sandoval & Cotman (1978). They showed that cerebellar synaptosomes exhibited calcium dependent release of glutamate. This release was most marked in synaptosomes prepared from the molecular layer (the region containing the granule cell axon terminals).

Climbing fibres powerfully excite the Purkinje neurons but their neurotransmitter is unknown. It is possible to destroy climbing fibres using 3-acetylpyridine (Desclin, 1974, Sotelo et al. 1975). In animals where these fibres have been destroyed the level of aspartate is significantly reduced (Nadi et al. 1977a). Since this compound is a putative excitatory neurotransmitter (see reviews by Curtis & Johnston, 1974; Krnjevic, 1974), Nadi et al. (1977a) suggested that aspartate might be the neurotransmitter released from climbing fibres. However no direct evidence concerning the neurotransmitter role of this amino acid has been forthcoming.

A population of noradrenergic fibres arising in the locus coeruleus is known to synapse on Purkinje neurons (Bloom et al. 1971;

Hoffer et al. 1973). These fibres are inhibitory and the effects of stimulation of these fibres are mimicked by iontophoretically applied noradrenaline and cyclic 3′, 5′-adenosine monophosphate (cyclic AMP) and blocked by β-adrenergic antagonists and prostaglandins E_1 and E_2 (Hoffer et al. 1969, 1971, 1973; Siggins et al. 197la,b). These results and others suggest that noradrenaline inhibits Purkinje cell firing by acting through β-adrenoceptors to stimulate adenyl cyclase (reviewed by Nathanson, 1977). (See page 72 for details of the various subclasses of adrenoceptors). This idea is supported by the finding that the number of Purkinje cells stained for cyclic AMP by immunohistological techniques, is increased following the application of noradrenaline (Siggins et al. 1973b). More recently Atlas et al. (1977) reported that it was possible to visualise the Purkinje β-adrenoceptors at the light microscope level using the fluorescent β-adrenergic probe 9-aminoacridine propranolol (9-AAP). These workers showed that fluorescence was restricted to the surface of these cells and was abolished by the presence of ℓ-propranolol. This is what one would predict if 9-AAP were binding to the Purkinje cell β-adrenoceptor (see section 1.7 for details of neurotransmitter receptor binding). However Hess (1979) has reported that 9-AAP binding is not as selective as Atlas et al. (1977) suggests and has shown that autofluorescence confounds these studies.

The neurotransmitter of the mossy fibres is unclear (Tebecis, 1974). However the distribution of enzymes for the synthesis (choline acetyltransferase) and degradation (acetylcholinesterase) of acetylcholine indicate that it may function as a neurotransmitter in the granule cell layer (Kasa & Silver, 1969; Shute & Lewis, 1965; Kan et al. 1978). These enzymes appear to be restrictied mainly to

the archicerebellar cortex in the rat. The electro-physiological data are conflicting. Crawford et al. (1966) observed that granule cells were unresponsive to acetylcholine or cholinomimetics. However McCance & Phillis (1968) observed that acetylcholine was capable of stimulating the deep granule cells, which along with the histochemical data concerning the localisation of acetylcholinesterase led them to conclude that a proportion of cerebellar mossy fibres was cholinergic. Neurotransmitter binding studies (see section 1.7) using muscarinic and nicotinic cholinergic ligands show that there are comparatively few acetylcholine receptors in this brain region (Yamamura & Snyder, 1974a; Yamamura et al. 1974; Kobayashi et al.1977; Segal et al. 1978). Of these, the concentration of muscarinic receptors is the highest. However, autoradiographic analysis of the distribution of these receptors reveals that in adult rat the majority of the muscarinic binding occurs in the molecular layer (Rotter et al. 1979b). However, this distribution pattern does not correspond to any known cholinergic input and unfortunately Rotter et al. 1979b) were not able to identify the precise cellular site of this binding. By contrast, autoradiographic analysis of ^{125}I-α-bungarotoxin binding revealed that the cerebellar glomeruli were heavily labelled by this nicotinic receptor ligand (Hunt & Schmidt, 1978). Thus it appears that if there are any cholinergic mossy fibres then they excite the granule neurons through nicotinic receptors on these cells. The role of muscarinic receptors in the adult cerebellum is unknown and Rotter et al.(1979b,c) has suggested that such receptors may be vestigial.

As mentioned in section 1.1, 5-HT containing fibres are present in the cerebellar cortex mainly in the granular layer. These fibres appear to terminate in structures resembling mossy fibre rosettes

(Bloom et al. 1972). Iontophoretically applied 5-HT excites the granule cells, and destruction of the raphe nuclei (the source of most 5-HT fibres) results in the degeneration of 5-HT containing fibres (Bloom et al. 1972); all of which suggests that certain of the mossy fibres may release 5-HT as a transmitter.

In summary: the neurotransmitters of the cerebellar inhibitory neurons (GABA) and a small proportion of axons synapsing on Purkinje cells (noradrenalin) are firmly substantiated. The evidence concerning the role of glutamate as the granule cell transmitter is conflicting and, with the exception of one study, circumstantial. This amino acid has also been suggested as the neurotransmitter of the climbing and mossy fibres although there is no direct evidence. Acetylcholine may function as the neurotransmitter of the mossy fibres. Other neurotransmitter candidates for which limited evidence is available are, taurine (neurotransmitter of superficial stellate cells), aspartate (climbing fibres) and 5-HT (mossy fibres).

1.5 Cell isolation techniques

The contribution of individual cell types to the overall functioning of the brain would be more easily studied if relatively pure populations of these cells were available. A number of workers have concentrated on obtaining cell fraction enriched in glial or neuronal cells (Poduslo & Norton, 1975; Rose, 1972; Sellinger et al. 1971). In this section a method for the isolation and separation of the major neuronal cell types and the glia from the cerebellum is introduced. The cerebellum is an ideal brain region from which to obtain enriched cell populations for the following reasons. The cerebellum contains only five major neuronal cell types and these cells have been well characterised (see section 1.1). The development of the neuronal and glial cells of the cerebellum has been well documented and this development occurs mainly postnatally in the rodent (see section 1.2 and Lewis et al. 1977).

Cell isolation is always associated with some degree of cell damage (Johnston & Roots, 1970; Rose, 1972) and although a number of workers have attempted to isolate enriched cell populations from the cerebellum (Sellinger et al. 1971; Cohen et al. 1973; Hazama & Uchimura, 1974; Yanigihara & Hamberger, 1973; Barkley et al. 1973), they did not extensively assess this damage; this problem has been examined by Poduslo & McKhan (1977) and Campbell et al. (1977).

In this laboratory a method has been devised (in collaboration with Dr. R. Balazs, Institute of Neurology, Queens Square, London) for the isolation of perikarya from the developing rat cerebellum (Wilkin et al. 1976). Close attention was paid to structural and biochemical integrity of these cells. The isolation technique involves mild trypsinisation

to facilitate the disaggregation of the tissue without shearing, a prominent feature of isolation featuring solely mechanical disruption. Following trypsinisation the tissue is disaggregated by passing it through a pasteur pipette (see Chapter 2 for detailed method).

This isolation technique resulted in high yields of viable cells, free of debris, from the developing rat cerebellum (345 x 10^6 cells/g wet wt from 10 day animals). According to the criterion of DNA recovery only 57 percent of the cells are lost during the isolation procedure. Ten days is the optimum age for cell isolation since in older animals fewer cells survive the procedure, possibly as a consequence of the more differentiated state of the cells in older animals. Details of the structural and metabolic integrity of these cells are as follows: over 80 percent of cells produced by enzymic disaggregation excluded trypan blue (indicating that the plasma membrane was intact) compared with less than 20 percent of cells produced by sieving. Electron micrographs of the enzymically treated cells showed that most of these cells had an uninterrupted plasma membrane and the cytoplasmic and nuclear ultrastructures were well preserved. In addition these electron micrographs of isolated cells were markedly similar to micrographs taken of cells in situ. The cells were metabolically active and were able to, metabolise (^{14}C) glucose, accumulate potassium and incorporate radio-labelled lysine into protein. However the most convincing demonstration of viability comes from culture studies. These perikarya can be readily cultured, and using the criterion of DNA recovery, 90 percent of the cells survive the first day in culture (Currie et al. 1979).

Advantage was taken of the wide variation in cell size seen in the cerebellum, and reflected in the isolated cells, to obtain enriched cell populations by unit gravity sedimentation (Cohen et al. 1978).

Briefly this consisted of layering the cells on top of a continuous bovine serum albumin gradient and allowing the cells to fall through the gradient (see Chapter 2 for details). The total metabolic activity of enriched cell populations was comparable to that seen for the original cell suspension. Four cell fractions (designated B-E) have been tentatively identified (see Table 1). The largest cells are enriched in the E fractions (i.e. 50 percent of the cells in this fraction are greater than 14.5 μm in diameter compared with 2 percent in the original cell suspension). Around 40 percent of these cells were identified as Purkinje cells in low power electron micrographs. The cells in the D fraction were not identified by Cohen et al.(1978). However Cohen et al. (1979) showed, using the glial marker anti-glial fibrillary acid protein (anti-GFAP) (see section 1.2), that astrocytes were enriched in this fraction. Currie & Dutton (unpublished observations) were able to show that a considerable proportion of cells cultured from this region resembled interneurons. Moreover these cells accumulated (^{3}H) GABA, which could be inhibited by cis-aminocyclohexanecarboxylic acid (ACHC), strongly indicating that these cells are GABAergic neurons (see section 1.6). (^{3}H) Thymidine is rapidly accumulated by cells in the C fraction and it was suggested (Cohen et al. 1978) that this fraction might be enriched in dividing granule cell precursors. The appearance of these cells, their size and their predominance in the total cell suspension are consistent with this suggestion. However there is an enrichment of GFAP-positive cells in these fractions and considerable numbers of glia are also probably present. The differentiating granule cells are thought to occur in the B fraction, since these cells do not accumulate (^{3}H) thymidine and they are similar in size and appearance to the premigratory granule cells. The C' fraction contains cells of the same size as the C cells but with anomolous sedimentation characteristics (they are lighter than

their size would indicate). Although the identity of these cells is not clear it is possible that they are damaged. It was suggested by Cohen et al. (1978) that these cells might be glia but the subsequent demonstration of only small numbers of GFAP positive cells in these fractions (Cohen et al. 1979) indicate that this is unlikely. The A fraction is subcellular and consists mainly of free nuclei and neuronal and glial fragments.

Isolated cells and enriched cell fractions, produced by modifications of the methods outlined above (Wilkin et al. 1976; Cohen et al. 1978), were used to study the transport of (^{3}H) GABA, (^{3}H) glutamate and (^{3}H) β-alanine into neurons and glia. (See section 1.6 for background to transport studies and Chapter 2 for detailed aims of the study).

A number of workers have tried to isolate the large synaptic specialisation peculiar to the cerebellar cortex, i.e. the cerebellar glomerulus (Israel & Whittaker, 1965; Hajos et al. 1974; Balázs et al. 1975; Hajos et al. 1975). The glomerulus consists of a mossy fibre terminal surrounded by the dendritic claws of granule neurons. In turn these dendrites are contacted by inhibitory Golgi axons (Palay & Chan Palay, 1974). These glomerular particles have been biochemically characterised (Hajos et al. 1974; Balázs et al. 1975) and are highly enriched in glutamate decarboxylase (GAP), the enzyme responsible for the synthesis of GABA. This is consistent with the role of GABA as the Golgi cell neurotransmitter (see section 1.4). The presence of Golgi terminals is also supported by the finding that the dendritic processes on the surface of the glomeruli accumulate (^{3}H)

GABA (Wilkin et al. 1974, see also section 1.6). As discussed in section 1.4, acetylcholine has been suggested as a neurotransmitter of certain mossy fibres. However there is only a slight enrichment of cholineacetyltransferase in the glomeruli suggesting that only a fraction, if any, of the mossy fibres are cholinergic.

Cerebellar glomeruli produced by a modification of the method of Hajos et al. (1975) were used here to examine neurotransmitter receptor binding (see section 1.7 for background to neurotransmitter binding and Chapter 3 for detailed aims of the study).

TABLE 1 Particle size analysis and tentative identity of fractions from unit gravity sedimentation procedure.

Fraction	Tentative identification of predominant cell types	Predominant particle size in fraction (Diameter uM)	Percent of cells (of indicated size range) in the total cell suspension.
A	Nuclei and subcellular debris*	5.5 - 6.5	7
C '	Cells with anomalous sedimentation characteristics* (damaged astroglia?)	8.0 - 10	Indistinguishable from C in the total cell suspension
B	Immature (premigratory) granule cells*	6.5 - 8.0	35
C	Dividing granule neuroblasts* and glial cells[b]	8.0 - 10	36
D	Inhibitory interneurons[a] and glial cells[b]	10 - 14.5	20
E	Purkinje cells*	14.5	2

* Cohen et al., 1978. [a]Currie & Dutton unpublished. [b]Cohen, Woodhams & Balazs (1979).

1.6 High affinity uptake of putative amino acid neurotransmitters

A number of amino acids including γ-aminobutyric acid (GABA) glycine, taurine, aspartate and glutamate have been proposed as neurotransmitters in the mammalian central nervous system (reviewed by Curtis & Johnston 1974). Of these amino acids the neurochemistry and neuropharmacology of GABA and glutamate have been the most widely studied (see section 1.4).

(^{3}H)GABA and (^{3}H)glutamate are taken up by high affinity, carrier-mediated, active transport systems in the brain, and it has been postulated that such systems may be responsible for the removal of released neurotransmitter from the synaptic cleft (Iversen & Neal, 1968; Logan & Snyder 1972). However, whether high affinity transport results in the net uptake is unclear, since a number of reports suggest that tritiated amino acid uptake may proceed by homoexchange (reviewed by Fagg & Lane, 1979). However the net uptake of glutamate into synaptosomes has been demonstrated by Roskowski (1978). In addition Roskowski (1978) has reported that net uptake of GABA takes place into synaptosomes, but only when they have been depleted of this neurotransmitter by potassium stimulated depolarisation.

GABA is the most firmly substantiated neurotransmitter candidate in the mammalian brain and is probably the neurotransmitter of the cerebellar Purkinje, stellate, basket and Golgi cells. (see section 1.4). High affinity transport of (^{3}H) GABA in rat brain was first reported by Iversen & Neal (1968), and subsequent autoradiographic studies indicated that in the cerebellum (^{3}H) GABA was rapidly accumulated by inhibitory neurons both *in vivo* (Schon & Iversen, 1972; Hokfelt & Ljungdahl, 1972) and *in vitro* (Sotelo *et al.* 1972; Ljungdahl *et al.* 1973; Lasher 1974; Hosli & Hosli 1976). Glia also possess a high affinity transport system for GABA (Schon & Kelly, 1974b; Schousboe et al. 1977a). However the

substrate specificity of the neuronal and glial transport systems is different (reviewed by Iversen & Kelly, 1975). Neuronal (^{3}H) GABA transport can be inhibited specifically by cis 3-aminocyclohexanecarboxylic acid (ACHC) (Bowery et al. 1976; Neal & Bowery, 1977) and glial (^{3}H) GABA uptake by β-alanine (Schon & Kelly, 1974b; Iversen & Johnston 1971). In the brain it is not possible to quantify glial uptake of (^{3}H) GABA by specifically inhibiting the neuronal component since glial transport is small by comparison (Schon & Kelly 1974b). However glial GABA transport can be examined using (^{3}H) β-alanine which is a specific substrate for this system (Schon & Kelly, 1975; Kelly & Dick 1976). (^{3}H) GABA uptake into glia also differs from neuronal uptake in that the former is markedly affected by the GABA: glutamate transaminase inhibitors such as aminooxyacetic acid (AOAA) (Iversen & Johnston 1971; Snodgrass & Iversen, 1974; Schon & Kelly 1974b). It is suggested that AOAA prevents the rapid degradation of (^{3}H) GABA by glia to readily excreted metabolites, thus increasing the apparent uptake of (^{3}H) GABA into these cells.

Compared to GABA the role of glutamate as a neurotransmitter is unclear. In the cerebellum a number of workers have suggested that glutamate may be the neurotransmitter released by the granule cell parallel fibres (reviewed in section 1.4).

(^{3}H) glutamate is taken up by high affinity active transport systems in rat synaptosome preparations and brain slices (Logan & Snyder 1972; Balcar & Johnston 1972). However the relative role of neurons and glia in the uptake of glutamate is disputed. Freshly prepared and cultured glial cells accumulate (^{3}H) glutamate by high affinity transport (Henn et al. 1974; Balcar et al. 1977; Faivre-Bauman et al. 1974; Schousboe et al. 1977b). In addition autoradiographic studies of brain, retina and spinal sensory ganglia demonstrate that glia, but not neurons,

accumulate (^{3}H) glutamate (Schon & Kelly 1974a; White & Neal 1976; McLennan 1976). However Weiler et al. (1979) has reported that glutamate uptake into synaptosomes is 3-5 fold higher than into glia, and Beart (1976) has presented autoradiographic evidence for uptake into synaptosomes. In addition, a recent autoradiographic study indicates that (^{3}H) glutamate is accumulated by nerve endings in the hippocampus (Storm-Mathisen & Iversen, 1979). Moreover (^{3}H) glutamate uptake in this region is attenuated by lesioning axons to this brain area (Storm-Mathisen, 1977).

Recent autoradiographic experiments in this laboratory show that in cultures of cerebellar neurons (^{3}H) glutamate is taken up by glial cells but not by granule cells (Currie & Dutton, unpublished) for which glutamate is a putative neurotransmitter.

In this thesis the uptake of (^{3}H) GABA, (^{3}H) β-alanine and (^{3}H) glutamate by cerebellar perikarys isolated from 10 day rat cerebellum (see section 1.4) was examined. See Chapter 2 for the detailed aims of the study.

Analysis of transport data. Carrier-mediated transport must by definition be saturable, and the transport of molecules by such a system can be characterised by two parameters i.e. the affinity of the transported molecule for the carrier (K_t) and the number of carriers. The latter parameter is generally expressed indirectly as the maximum rate of transport that the system can achieve. An equation which describes binding in terms of these parameters can be derived thus:

$$C + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} CS \xrightarrow{k_2} C + S$$

where S = substrate

C = carrier

CS = substrate carrier complex.

The assumption is made that transport is relatively slow compared with the formation of the complex. Hence the complex is in equilibrium with the substrate and carrier, i.e.

$$C + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} CS$$

Therefore according to the Law of Mass Action:

$$k_1 . (C) (S) = k_{-1} . (CS) - (i)$$

but $(C) = (C_{total}) - (CS) - (ii)$

substituting equation (ii) in (i).

$$k_1 . (S) . ((C_{total}) - (CS)) = k_{-1} . (CS) - (iii)$$

re-rranging equation (iii).

$$(CS) = \frac{(Ctotal).(S)}{(S) + \frac{k_{-1}}{k_1}} \quad -(iv)$$

but transport (v) is given by:

$$v = (CS).k_2 \quad \therefore \quad (CS) = \frac{v}{k_2} \quad -(v)$$

substituting equation (v) in (iv).

$$\frac{v}{k_2} = \frac{(Ctotal).(S)}{(S) + \frac{k_{-1}}{k_1}} \quad -(vi)$$

rearranging equation (vi).

$$v = \frac{k_2.(Ctotal).(S)}{(S) + \frac{k_{-1}}{k_1}} \quad -(vii)$$

Maximal rate of transport is given by:

$$k_2.\ (Ctotal) = V \quad -(viii)$$

and the affinity of the substrate for the carrier is given by:

$$\frac{k_{-1}}{k_1} = K_t \quad -(ix)$$

substituting equations (viii) and (ix) in (vii).

$$v = \frac{V.\ (S)}{K_t + (S)} \quad -(x)$$

This is identical to the Michaelis-Menten equation which was initially derived to describe the catalysis of biochemical reactions by enzymes (see Neame & Richards, 1972 for further background to transport kinetics). The Michaelis-Menten equation describes a rectangular hyperbola and when plotting experimental data the Eadie Hofstee transformation of equation (x) which describes a straight line, is used.

$$\text{i.e.} \quad v = -Kt.\left(\frac{v}{(s)}\right) + V$$

Thus plotting v against $\frac{v}{(S)}$, $-K_t$ is given by the slope of the line and V by the y intercept.

Analysis of transport data. Following the incubation of tissue with the radiolabelled substrate the excess substrate must be removed and there are two methods routinely used. The tissue can be filtered and washed over glass-fibre or paper filters under slight suction, which was the method used in this study. Alternatively the tissue can be centrifuged and the excess ligand removed by aspiration. This method was used mainly when examining uptake into isolated cells or synaptosomes. The relative merits of these two methods are discussed in section 1.7 and will not be dealt with here.

Although the excess substrate is removed some radioactivity will remain which is not the result of transport. Some of the substrate will enter the tissue by diffusion. If the centrifugation method is used a significant amount of the substrate will remain trapped in the water space in the pellet. Even using a filtration assay some of the substrate will remain with the tissue and on the filter as a result of

physicochemical attraction phenomena. Such remaining radioactivity is often referred to as non-specific binding (see also section 1.7).

In assays using the centrifugation technique (^{14}C) inulin or sucrose, which are not transported, have been used to determine the volume of the trapped water space (Henn & Hamberger, 1971; Levi & Raiteri, 1973; Somoza & De Feudis, 1978). The amount of trapped substrate can then be calculated. However this correction method does not account for diffusion or non-specific binding. Using the filter assay relatively little of the substrate is trapped especially when using cell suspensions as outlined in Chapter 2. However diffusion and non-specific binding must account for some of the apparent transport. A number of techniques have been employed to determine that uptake which is due to transport. A number of studies have used the apparent uptake which occurs at zero incubation time as a control (Iversen & Neal 1968; Balcar & Johnston 1972). Such controls give an estimate of non-specific binding, but not diffusion. An alternative is to perform parallel incubations at $0^{o}C$ (Schon & Kelly 1974; Campbell & Shank 1978; Thomas & Redburn 1978). Since high affinity transport of amino acids is an active process (Iversen & Neal 1968; Logan & Snyder 1972) transport should be markedly reduced at this temperature. Thus an estimate of diffusion and non-specific binding can be obtained. However Iversen and Neal (1968) have shown that significant (^{3}H) GABA transport occurs even at these low temperatures. Since transport is saturable the addition of cold substrate will reduce the amount of labelled substrate taken up. If the concentration of radiolabelled substrate is close to, or less than, its dissociation constant (K_t),

then the presence of cold substrate at a concentration of 100-1000 fold greater than the K_t will result in 98-100 percent inhibition of uptake. Thus the difference in uptake in the presence and absence of excess cold substrate will be a measure of transport. This correction method has been used in this and other transport studies (Olsen et al. 1978), and it gives an estimate of both non specific binding and diffusion.

The detailed aims of studies involving the uptake (^{3}H) GABA, (^{3}H) glutamate and (^{3}H) β-alanine by isolated cerebella perikarya (see section 1.4) are outlined in Chapter 2.

1.7 Neurotransmitter receptor binding

Prior to 1965 the examination of neurotransmitter receptors was restricted to dose-response studies. In the brain this generally involves the use of iontophoresis which has a number of limitations, not least of which is the difficulty in determining the precise concentration of a ligand ejected from a pipette, at the site of action. In 1965 Paton & Rang published an account of the binding of ^{3}H-atropine to muscarinic receptors in the guinea pig ileum. Because the specific activity of the ligand was relatively low and specific binding was only a small fraction of total binding, detailed characterisation of the receptor was not possible. Interest in the labelling of neurotransmitter receptors in the central nervous system was stimulated by the discovery of opiate binding receptors in the brain (Pert & Snyder, 1974; Simon et al. 1973; Terenius, 1973). Since that time, ligands of high specific activity have become widely available for a number of postulated neurotransmitter receptor systems (see Snyder & Bennet, 1976; Wolf et al. 1977; Maguire et al. 1977; Yamamura et al. 1978, for reviews and Table 2 for a list of the ligands commonly in use).

Binding will occur between all radioligands and biological material. However a proportion of this binding will be non-specific, (i.e. binding not associated with the receptor) and this binding is generally non-saturable. The causes of non-specific binding include such phenomena as entrapment of the ligand by the tissue, ionic and hydrophobic interactions, and van der Waal's forces. To determine the amount of non-specific binding controls are included in the assay which contain excess cold ligand or other displacer (discussed further

below), which competes for the specific binding sites, excluding the radioligand. However the presence of excess ligand does not alter the number of radioactive molecules bound to non-specific sites. Thus the difference between binding of radioligand in the presence and absence of a displacer is taken to be specific binding. Although the displacement of a radioligand in this way meets the first criterion for the binding of a molecule to a receptor, i.e. saturability of the receptor is confirmed, this cannot be taken as proof of binding to a biological receptor. Cuatrecasas & Hollenberg (1975) demonstrated the saturable binding of ^{125}I-insulin to talcum powder, and Snyder et al. (1975b) were able to show stereospecific saturable binding to glass fibre filters commonly used in neurotransmitter receptor assays. These examples serve as a reminder that saturable binding is not exclusively a biological phenomena.

In order to be confident that a ligand is binding to physiological receptors the following criteria (which are not exhaustive) must be fulfilled.

(i) Binding must be saturable, stereospecific and of high affinity. The binding parameters should also match what is known about the receptor from physiological studies. For example, the biologically active stereoisomer should be bound, the biological potency of the ligand should reflect the binding dissociation constant and binding sites should be of an appropriate number. Generally neurotransmitter receptors have a density range of 10-100 pmoles/g tissue (Snyder & Bennet, 1976).

(ii) The radioligand should be displaced by drugs known to act through the receptor to which the ligand is thought to be bound. For example (^{3}H) QNB binding which is thought to bind to the muscarinic cholinergic receptor is displaced by both muscarinic antagonists and agonists but not by nicotinic drugs or other neurotransmitter candidates or their analogues (Yamamura & Snyder, 1974a). In addition the in vivo potency of drugs should reflect their ability as displacers. This generally holds true in that the rank order of antagonists biological potency matches their ability to displace ligands and similarly for agonists. However major discrepancies are seen between biological and binding potency when agonists are used to displace antagonists and vice versa. For example acetylcholine is not as effective at displacing (^{3}H)QNB as one might predict from its biological activity (Snyder et al. 1975a). To account for these phenomena it has been proposed that the receptor exists in two forms (which may or may not be interconvertible). Each form binds the agonist or antagonist preferentially. Receptor systems for which these models have been postulated include dopamine (Burt et al. 1976) serotonin (Bennet & Snyder, 1976), the opiates (Pert & Snyder, 1974), adrenergic system (U'Prichard et al. 1977). A similar two site model has been postulated to account for the binding properties of muscarinic receptors (Birdsall & Hulme, 1976a) and opiate receptors (Birdsall et al. 1976b). However in this model the two sites are postulated to have identical affinities for antagonists, but one site has a higher affinity for agonists.

(iii) Since neurotransmitters are thought to act as receptors on the cell surface, binding should be highly enriched in preparations containing external cell membranes and in particular synapses. Also if the radioligand can be visualised by autoradiography one would

expect to see the ligand concentrated at cell surfaces. (^{3}H)QNB binding for example is enriched in the microsomal pellet which includes the synaptosomes (Yamamura & Snyder, 1974a). Also using autoradiographic techniques Chan-Palay (1978) was able to show that binding appeared to be localised on cells, adjacent to areas of synaptic contact.

(iv) The density of innervation of a brain region (or organ) might be expected to correlate with the density of binding sites for an appropriate neurotransmitter in that area. However in the brain the density of innervation is generally determined by the level of presynaptic markers. These parameters only give an indication of the number of axons in an area and not the number of nerve terminals. Also the number of receptors need not necessarily be related to the number of terminals, it might for example, be more dependent on the number of receptive neurons in the area. Where a neurotransmitter is known to be of importance in a particular brain region there is often a higher concentration of receptors in that area. For example, GABA is known to be the major inhibitory transmitter in the cerebellum (see section 1.4) and this brain region contains the highest concentration of GABA receptors (Zukin et al. 1974).

As already mentioned the criteria for receptor identification outlined here are not exhaustive and a more complete list is given by Burt (1978b).

Kinetics of binding. The binding of a radioligand to its receptor generally obeys the law of mass action although there are

notable exceptions to this rule (Burgen, Hiley & Young 1974; Birdsall et al. 1976 a; Birdsall & Hulme 1976; Limbird & Lefkowitz, 1976). Saturable binding can be characterised by two parameters; the strength of attachment between the ligand and its receptor, i.e. the association constants, or its reciprocal, the dissociation constant (Kd) and the number of sites available for binding, designated Bmax.

A straight line equation for the binding of a ligand to its receptor can be derived thus:

$$F + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} RF \qquad -(i)$$

where F = unbound ligand

R = free receptors

RF = ligand bound to receptors

if B = amount of ligand bound to receptor, under equilibrium conditions -

$$R = B_{max} - B$$

substituting this information in equation (i)

$$F + (B_{max} - B) \underset{k_2}{\overset{k_1}{\rightleftharpoons}} B$$

thus according the Law of Mass Action, at equilibrium

$$K_d = \frac{F\ (B_{max}-B)}{B} \qquad -(ii)$$

rearranging equation (ii) gives

$$B/F = (B_{max} - B)/K_d$$

This is the Scatchard (1949) equation which is identical to the Eadie Hofstee transformation of the Lineweaver Burk equation for the interaction between an enzyme and its substrate, i.e.

$$\frac{v}{(\bar{s})} = \frac{V-v}{Km}$$

Thus by plotting binding data as B/F against B the experimental points should fit a straight line, the slope of which will yield -1/Kd and x intercept B_{max}. Normally the amount of ligand is not depleted significantly as a result of binding. Free ligand therefore is usually taken to mean the ligand concentration at the start of the experiment. However if the amount of ligand bound is a significant amount of the free ligand this must be taken into account when constructing the Scatchard plot. An additional check that binding obeys the Law of Mass Action can be made by expressing the data as a Hill plot (see Birdsall & Hulme, 1976) i.e. plotting B/B_{max}-B against log (S). The gradient of such a plot (the Hill coefficient nH) should equal unity. A number of workers have reported Hill coefficients of significantly less than one for the binding of tritiated muscarinic agonists (reviewed by Birdsall & Hulme, 1976) and the β-adrenergic antagonist (^{3}H) dihydroalprenolol (^{3}H)DHA Limbird & Lefkowitz, 1978 - though see later in this section for details of experiments involving (^{3}H)DHA binding. There are a number of explanations which would account for such deviations of the Hill coefficient from unity. These include: negative cooperativity, i.e. the binding of the ligand brings about a conformational change in adjacent receptors reducing their affinity for the ligand. The

desensitisation of receptors by the ligand would also account for this phenomenon. However in the case of acetylcholine agonists the most likely explanation was thought to be the presence of heterogeneous receptor populations (Birdsall & Hulme, 1976).

Estimation of non-specific binding. The addition of excess cold ligand in neurotransmitter binding assays must by definition reduce the binding of the radioligand if binding is saturable. However one must be confident that the displacer is removing most of the ligand bound to the receptors. If the concentration of radioligand used is close to its Kd then 100-1000 fold excess of cold ligand will displace 98-100 percent of the specifically bound counts. Often a displacer different from the radioligand is used in an attempt to increase the likelihood that true receptor binding is being characterised. In such circumstances a concentration of displacer 40-fold higher than its IC_{50} (the concentration of displacer required to reduce specific binding of the radioligand to 50 percent control) will result in 98 percent displacement of specific counts.

Separation of the tissue from the incubation medium. There are two major methods available for separating the tissue from the excess radioligand: rapid filtration under suction through glass fibre filters, followed by rapid rinsing of the filters with buffer, or rapid centrifugation of the incubation followed by superficial rinsing of the pellet. The first method is suitable only for ligands which are relatively tightly bound to the receptor and which will not be removed in significant amounts from the receptors during the 30 seconds it takes to complete the procedure. This

is the method employed for the study of (^{3}H) dihydroalprenolol ((^{3}H) DHA) and (^{3}H) quinuclidinylbenzilate ((^{3}H) QNB) in this study. The centrifugation method is used for those ligands which rapidly dissociate from their receptors and are likely to be "washed off" if a filter assay were used. Where appropriate, the filter method is by far the superior of the two methods since it is rapid and the washing of the tissue and filter markedly reduces non-specific binding. Non-specific binding is particularly high when the centrifugation assay is used since inevitably some unbound ligand remains trapped in the water space of the pellet (see Bennett, 1978, for further discussion).

Muscarinic acetylcholine receptor. Tritiated acetylcholine (which also binds to nicotinic receptors) and its more specific agonists have been used to label these receptors (Birdsall et al. 1976a). However antagonists are generally used to label these receptors since they bind with much higher affinity. This allows the use of low ligand concentrations, which results in lower non-specific binding. Thus the level of specific binding can be more accurately determined. Such antagonists include (^{3}H) atropine, (^{3}H) QNB (^{3}H) dexetimide and the irreversible antagonist (^{3}H) propylbenzilylcholine mustard (PBCM) (Paton & Rang, 1965; Yamamura & Snyder, 1974a,b; Laduron et al. 1979; Burgen et al. 1974). Of these compounds only (^{3}H) QNB is widely available. (^{3}H) QNB binding to muscarinic cholinergic receptors was first described by Yamamura & Snyder (1974a).

This ligand fulfills all the criteria for binding to a receptor outlined earlier in this section (reviewed by Snyder et al. 1975a;

Birdsall & Hulme 1976). Binding is of very high affinity (Kd = 0.4 nM) and is suitable for study by filtration assays (Yamamura & Snyder, 1974a). (^{3}H) QNB has been used extensively to study muscarinic receptors in central and peripheral nervous tissues (Yamamura et al. 1974; Yamamura & Snyder, 1974a; Kobayashi et al. 1978; Yavin & Harel, 1979; Ben-Barak & Dudai, 1979; Burt, 1978b; Sugiyama et al. 1977). Binding has also been examined in cultured neurons and a variety of tissues including heart and gut (Strange et al. 1978; Dudai & Yavin, 1978; Siman & Klein, 1979; Galper & Smith, 1978; Roeske & Yamamura, 1978; Yamamura & Snyder, 1974b).

As already discussed the binding of muscarinic agonists and antagonists is consistent with binding to two receptors which have identical affinities for the antagonist but differing agonist affinities (Birdsall & Hulme, 1976). The relevance of these sites is unclear, although they may be related to different receptor-effector mechanisms (Birdsall et al. 1976a). However antagonist ligands can be used to label both forms of the receptor.

β-Adrenergic receptor. Just as acetylcholine receptors can be subdivided into muscarinic and nicotinic receptors, so the adrenergic receptors can be classified as α and β-adrenoceptors on the basis of antagonist specificity (Nickerson & Collier, 1975). The picture is further complicated by the finding that the β-adrenoceptors can be further subdivided into B_1 and B_2 receptors on the basis of their sensitivity to selective antagonists and the potency of agonists (Lands et al. 1966, 1967; Koelle, 1975).

For similar reasons to those outlined for the use of muscarinic antagonists, β-adrenergic antagonists are generally used in receptor binding studies (see reviews, Wolfe et al. 1977; Maguire et al. 1977). Two ligands are routinely used and commercially available; (^{3}H) DHA and (^{125}I) iodohydroxybenzylpindolol ((^{125}I)IHYP). Although (^{125}I)IHYP binds with a higher affinity and is available at a much higher specific activity than (^{3}H) DHA, the latter ligand is often preferred because it is the more stable of the two ((^{125}I)IHYP breakdown can occur within minutes) and the specific activity of the compound does not need constantly redetermining (Maguire et al. 1977).

(^{3}H) DHA binding to rat brain tissue has been characterised by Alexander et al. (1975) and Bylund & Snyder, 1976 and these studies indicate that the compound fulfills all the criteria required of a specific β-adrenergic ligand. Binding is of high affinity (Kd=1nM) and, like (^{3}H) QNB, this ligand is suitable for filter assays (Byland & Snyder, 1976). (^{3}H) DHA has been used extensively to examine β-adrenoceptors in a variety of structures and systems including brain, heart, liver, cultured cells and erythrocytes (reviewed by Maguire et al. 1977; Wolfe et al. 1977).

Both DHA and IHYP are, like many β-adrenergic antagonists, hydrophobic molecules, which may account for the local anaesthetic properties of these compounds (Maguire et al. 1977; Moe & Abildskov, 1975). If one uses one β-antagonist to displace another (e.g. propranolol to displace (^{3}H) DHA) it has been demonstrated that a second low affinity binding site is revealed (Bylund, 1978; Nahorski & Richardson, 1979; Mendel & Almon, 1977). It has been suggested that this displacement of the radioligand from a low affinity site

is due to the local anaesthetic action of these compounds. Thus the addition of an excess of displacer alters the partition coefficient of the radioligand, resulting in displacement of the ligand from the tissue. (Mendel & Almon, 1977). If instead of an antagonist one uses an agonist such as isoprenalin, which is devoid of local anaesthetic effects, then displacement is consistent with (^{3}H) DHA binding to a single population of receptors (Bylund, 1978; Nahorski & Richardson, 1979). As one might expect the low affinity site is not stereospecific, since it is a simple physicochemical interaction of the ligand with the tissue, and the ligand is displaced by antagonists even in the presence of excess isoprenalin (Nahorski & Richardson, 1979). Limbird & Lefkowitz (1976) have reported that (^{3}H) DHA binding displays negative cooperativity. However, these authors used propranolol as a displacer, and since the Scatchard plots of Nahorski & Richardson (1979) for (^{3}H) DHA binding displaced by propranolol resemble negative cooperativity curves, it is possible that Limbird & Lefkowitz (1976) were observing the combined displacement of (^{3}H) DHA from low and high affinity binding sites. Whether two true β-adrenoceptors exist similar to that proposed for (^{3}H) QNB is unclear, although U'Prichard et al. (1978) have reported that (^{3}H) adrenaline is more readily displaced by β-agonists than antagonists. It should perhaps be noted here that (^{3}H) DHA binds to both B_1 and B_2 receptors (U'Prichard et al. 1978) and while it appears that β-receptors of the cerebellum are of the B_2 type, those in the cerebral cortex appear to be of the B_1 type (U'Prichard et al. 1978; Cote & Kebabian, 1978).

GABA receptor. This receptor has been studied using the endogenous ligand (^{3}H) GABA. Kinetic studies in the presence of sodium revealed two binding sites. The low affinity site was sodium-dependent, and binding was inhibited by GABA transport inhibitors suggesting that

this was the GABA transport receptor. However the high affinity site (Kd ≈ 100 - 400nM) is sodium independent (assays for this receptor are generally performed in the absence of sodium to reduce binding to the transport receptor) and fulfills all the criteria for GABA binding to its neurotransmitter receptor (Zukin et al. 1974; Enna & Snyder, 1975; Young et al. 1976). Unlike the other two receptor systems outlined (i.e. muscarinic-cholinergic and β-adrenergic receptors) there is not a wide range of specific GABA antagonists available from which to prepare radioligands. Neurophysiological studies indicate that bicuculline is a relatively specific GABA antagonist (Curtis et al. 1971; Johnston et al. 1972). Mohler & Okada (1977, 1978) have reported stereospecific high affinity binding of (^{3}H) bicuculline methiodide in rat brain. This binding was localised in synaptosomal preparations and was displaced by GABA and compounds thought to act at the neurotransmitter receptor, but not by GABA uptake inhibitors. However specific bicuculline binding can only be demonstrated in the presence of anions, such as thiocyanate, which promote protein unfolding (Collins & Cryer, 1978). In addition these anions increase ten fold the potency of bicuculline in displacing (^{3}H) GABA binding (Enna & Snyder, 1977). These findings suggest that bicuculline does not bind to exactly the same site as GABA (Collins & Cryer, 1978; Enna & Snyder, 1977). Other problems associated with the use of bicuculline for the study of GABA receptors are the relative instability of bicuculline and its methiodide derivative (although the methochloride is fairly stable). The affinity of binding of these compounds is not markedly greater than that of GABA.

There are a number of useful GABA agonists which bind with relatively high affinity. Of these (^{3}H) muscimol has been the most widely studied. This compound binds with a much higher affinity than GABA (Kd = 2-40 nM) and binding fulfills all the criteria for binding to the GABA neurotransmitter receptor (Beaumont et al. 1978; Snodgrass, 1978; Leach & Wilson, 1978; Wang et al. 1979). In addition, (^{3}H) muscimol resembles sodium independent (^{3}H) GABA binding in that it is enhanced by freezing and detergents (Enna & Snyder, 1977; Wong & Horng, 1977; Beaumont et al.1978; Snodgrass, 1978; Enna et al. 1979; Wang et al. 1979). The reasons for this enhancement is unclear, but there is evidence to suggest that freezing and detergents may remove an endogenous GABA receptor modulator (Guidotti et al. 1978; Johnson & Kennedy 1978). Displacement of (^{3}H) GABA and (^{3}H) muscimol by GABA agonists, and enhancement of the binding of these ligands following freezing and detergent treatment suggest that perhaps there is more than one GABA receptor, muscimol has a higher affinity for one of these sites (Enna et al. 1978; 1979; Herschel & Baldessarini, 1979). A comparison of (^{3}H) GABA and (^{3}H) muscimol binding in various brain regions indicate that the levels of binding are very similar (Enna et al. 1978). Moreover, the maximal binding levels are almost identical (Enna et al. 1979), indicating that there are the same number of muscimol sites as GABA sites. However, de Feudis et al. (1979a) claim to have shown that there are significantly more muscimol receptors than GABA receptors. Although (^{3}H) muscimol binding is generally examined using a centrifugation assay, a filtration assay for this ligand has been recently reported (Williams & Risley, 1979).

Localisation of neurotransmitter receptors. Although this section and the studies outlined in Chapter 3 deal solely with methods for the quantitative analysis of neurotransmitter binding, a number of workers have developed methods for visualising the distribution of binding using autoradiographic or fluorescence techniques (reviewed by Kuhar, 1978). Briefly, ligands are administered to living animals systemically, the animals are then killed and the brain tissue rapidly fixed. This technique has been used to examine the distribution of a number of receptors including GABA receptors using (^{3}H) muscimol (Chan-Palay, 1978; Chan-Palay et al. 1978), muscarinic receptors ((^{3}H)QNB) (Kuhar & Yamamura, 1975) and β-adrenoreceptors using the fluorescent ligand 9-AAP (Melamed et al. 1976). An alternative method is to incubate tissue slices in the presence of the ligand which allows greater control of the conditions under which binding occurs. This method has been used to label nicotinic receptors (^{125}I-α-bungarotoxin) (Segal et al. 1978) and muscarinic receptors ((^{3}H)PBCM) (Rotter et al. 1977; Rotter et al. 1979a). Using autoradiographic techniques it is possible not only to examine localisation by light microscopy, but also to resolve localisation at the subcellular level by electronmicroscopic autoradiography. The latter technique has been applied to the examination of nicotinic receptors at the synaptic junction using ^{125}I- -bungarotoxin (Fertruck & Saltpeter, 1974) and GABA receptors in the central nervous system using (^{3}H) muscimol (Chan-Palay et al. 1978; Chan-Palay, 1978; Chan-Palay & Palay, 1978). As in the other binding studies, binding to receptors is taken to be that displaced by excess cold ligand or other displacer (see also Kuhar, 1978) for other criteria necessary for receptor localisation.

Table 2 Some of the commercially available radioligands and the receptor systems to which they bind.

Ligand	Receptor	Displacer	Reference
(^{3}H) Quinuclidinylbenzilate (QNB)	Muscarinic acetylcholine	Atropine	Kobayashi et al. 1978)
(^{125}I) Iodo-α-bungarotoxin	Nicotinic acetylcholine	Nicotine	Segal et al. (1978)
(^{3}H) GABA	GABA	GABA	Enna & Snyder (1975)
(^{3}H) Muscimol	GABA	GABA	Snodgrass (1978)
(^{3}H) Dihydroalprenolol (DHA)	β-adrenergic	Isoproterol	Bylund (1978)
(^{125}I) Iodohydroxybenzylpindolol (IHYP)	β-adrenergic	Propranolol	Sporn & Molinoff (1976)
(^{3}H) Noradrenalin	α-adrenergic	Oxymetazoline	U'Prichard & Snyder (1977)
(^{3}H) Dihydroergokrypt (DHE)	α-adrenergic	Noradrenalin	Greenberg & Snyder (1977)
(^{3}H) Strychnine	Glycine	Glycine	Young & Snyder (1973)
(^{3}H) Spiroperidol	Dopamine	Butaclamol	Fields et al. (1977)
(^{3}H) 5-Hydroxytryptamine (5-HT)	5-HT	5-HT	Snyder & Bennet (1975)
(^{3}H) Lysergic acid diethylamide (LSD)	5-HT	LSD	Whitaker & Seeman (1978)

CHAPTER 2

TRANSPORT OF GABA, β-ALANINE AND GLUTAMATE INTO PERIKARYA OF RAT POSTNATAL CEREBELLUM

2.1 Introduction

In sections 1.5 and 1.6 the uptake of glutamate and GABA into neurons and glia was outlined and a method for the isolation and separation of perikarya from the developing rat cerebellum was described. In this Chapter the uptake of these amino acids into perikarya obtained by a modification of the method outlined in section 1.5 is examined in order to determine the following. As mentioned previously (section 1.5) cell isolation techniques severely disrupt the cell surface, and it would be useful to know whether newly isolated perikarya possess high affinity transport sites and of what type. In addition the uptake of β-alanine, GABA and glutamate by enriched perikaryal fractions obtained by unit gravity sedimentation has been examined in order to further characterise these fractions and to determine which population of cells is responsible for the uptake of glutamate.

2.2 Materials and Methods

Cell isolation and separation procedures

Cerebella from Wistar CFHB rat pups aged 10-11 days were used throughout. These animals were killed by decapitation and the cerebellum removed. The meninges were removed from the cerebellum using watch-makers forceps and 3-4 cerebella were used in the isolation

procedure. Isolated cells were prepared using a modification (Dutton, Currie & Tear, 1980) of the method of Wilkin et al. (1976). The modified method was as follows. All media were based on calcium and magnesium free Earles basic salt solution (Gibco-Biocult) containing in addition 0.3 percent bovine serum albumin (BSA, fraction V, Sigma), 14 mM glucose and 1.5 mM $MgSO_4$. (The glucose was added to the medium to provide a substrate for cell metabolism during the isolation and the presence of magnesium sulphate was required as a cofactor for DNase I used in the procedure). The medium was sterile filtered (0.22 µm, Millipore membranes) and maintained at physiological pH by gassing with 95 percent O_2 and 5 percent CO_2. All operations were carried out at room temperature unless specified otherwise. The cerebella were chopped at 400 µm intervals in two directions (the second pass being at right angles to the first) using a McIlwain chopper (Mickle Engineering Company). Chopped cerebella were dispersed in 10 ml of medium containing 0.025 percent trypsin (type III, Sigma) and incubated for 15 minutes at 37°C in a shaking water bath. (Brief tryptic digestion appears to reduce the degree of cell damage caused by cell isolation, presumably by breaking down the matrix which binds the cells together). The incubation was terminated by the addition of 10 ml of medium containing 6.4 µg per ml DNase I (Sigma) and 80 µg per ml soya bean trypsin inhibitor (SBTI, Sigma) and the tissue was sedimented. (The DNase reduces the tendency of the isolated cells to reaggregate due to the presence of chromatin released from damaged cells. Any further digestion of the cells would be undesirable and the SBTI prevents this). The supernatant was discarded and the pellet resuspended in 2 ml of medium containing 40 µg per ml DNase I, 0.5 mg per ml SBTI and 3mM magnesium sulphate.

The tissue blocks were sheared by trituration (12-15 times) using a siliconised Pasteur pipette. After 3-5 minutes the supernatant, containing mainly isolated cells, was transferred to a 10 ml conical centrifuge tube. The trituration procedure was repeated on the remaining undisrupted material and the supernatants combined. (Light-microscopic examination of the isolated cells reveals that these cells are shorn of their processes). Perikarya largely debris free, were obtained by underlaying the supernatant with 2 ml of medium containing 4 percent BSA and centrifuging (5 minutes, 100 xg). Pelleted cells were resuspended in 2-3 ml of calcium and magnesium free Krebs Ringer buffer or for the sodium dependence experiments in 0.32 M sucrose. Cell counting and sizing were performed using a Coulter Counter **2**B1 (Coulter Electronics) with a 140 µm orifice tube. The size ranges examined (designated A-E; see also Table 1) were approximate to the spectrum of cell sizes seen in the intact cerebellum (Wilkin et al. 1976). The instrument was calibrated with latex beads of known diameter. Although the isolated cells are damaged by the above isolation procedure (i.e. the removal of cell surface proteins by trypsin, and shearing of cell processes) the study of Wilkin et al. (1976) indicate that over 80 percent of these cells have intact cell membranes. Furthermore the isolated cells are metabolically active, synthesise proteins, accumulate potassium ions and are capable of long term survival in culture (Wilkin et al. (1976); Currie et al. (1979)) (see section 1.5 for further details).

Analysis of transport

General considerations. The uptake of 4-amino-n-(2,3-^{3}H) butyric acid ((^{3}H) GABA), β-(3-^{3}H) alanine ((^{3}H)β-alanine) and 1-(G-^{3}H)

glutamic acid (^{3}H) glutamate) (specific activities; 54, 49 and 30 Ci per mmol, respectively, all 1 µCi per µl, Radiochemical Centre Amersham) was measured using a filtration method as follows. Incubations were carried out in plastic-ware or siliconised glassware and unless specified otherwise the incubation temperature was 25°C. Triplicate 50-100 µl aliquots of cells were added to filtration stacks holding 5-10 ml of 0.9 percent saline. The cells were collected on glassfibre filter discs (Whatman GF/C by slight suction and washed a further 3 times with 5-10 ml of saline. The filters were treated with 150 µl of NCS (G.D. Searle Limited) to solubilise the tissue and the radioactivity was counted by liquid scintillation spectroscopy on a Beckman LS250 counter. The scintillant used was toluene methoxyethanol (4:1) containing 6g per l PPO. Correction was made for quenching by the external standards ratio technique (Peng, 1977). Unless stated otherwise correction was made for radioactivity not due to transport by performing parallel incubations in the presence of 1 mM GABA or 1 mM glutamate as appropriate or by removing aliquots of the incubate immediately after commencing the incubation (see section 1.6 for theoretical background to these corrections). In both cases counts not due to high affinity transport were typically less than 10% of the total counts.

Time-course of (^{3}H) GABA and (^{3}H) glutamate transport into the cell.

Two hundred microlitres of the cell suspension (2-11 x 10^6 cells) were incubated with 2 ml of Puck's saline G (PSG, Puck et al.1958) containing 20 µl of (^{3}H) GABA (final concentration 0.17 µm). This medium has been used previously for the examination of (^{3}H) GABA uptake into cultured neurons and glia (Lasher, 1974) and has advantage over EBSS in that it does not require gassing. The incubations were

carried out at 25° or 37°C and in the presence or absence of 10 µM aminooxyacetic acid (AOAA, Sigma). Aliquots were taken for analysis immediately after adding the cell suspension and at time intervals up to 32 minutes. The uptake of (^{3}H) glutamate (10 µCi in 2 ml final concentration 0.16 µM) was performed similarly except that AOAA was not used and uptake was examined at 25° only.

Dependence of (^{4}H) GABA and (^{3}H) glutamate transport on cell concentration. Twentyfive to one hundred microlitres of the cell suspension (1-7 x 10^{6} cells) were incubated with 10 µCi of (^{3}H) GABA or 5 µCi of (^{3}H) glutamate in PSG to give a final volume of 1.1 ml. Aliquots were removed from the incubates for analysis after 4 minutes.

Sodium dependence of (^{3}H) GABA transport. Two hundred microlitres of the cell suspension in 0.32 M sucrose (8 x 10^{6} cells) were incubated with 20 µCi of (^{3}H) GABA (final concentration 0.17 µM) in 2 ml of 0.32M sucrose. Triplicate 100 µl aliquots were taken from the incubate at 0, 2 and 4 minutes. After 4 minutes 1 ml of 0.9 percentNaCl was added to the incubate and further aliquots were taken at 6 and 8 minutes.

Kinetics of (^{3}H) GABA and (^{3}H) glutamate uptake. One hundred microlitres of the cell suspension were incubated with 1 ml of PSG containing (^{3}H) GABA and unlabelled GABA to a final specific activity of 1 Ci/mmol and a concentration range of 0.1-3.6 µM. Aliquots were taken for analysis after 4 minutes. In the case of (^{3}H) glutamate, 50 µl aliquots of the cell suspension were incubated with 0.5 ml of PSG containing varying amounts of (^{3}H) glutamate and unlabelled glutamate to give a final specific activity of 0.1 Ci/mmol and a concentration range of 2.5 - 100 µM. Aliquots were taken for analysis after 4 minutes.

Effects of inhibitors on (^{3}H) GABA transport. One hundred microlitres of cell suspension (2-5 x 10^6 cells) were pre-incubated for 4 minutes with 1 ml of PSG containing varying concentrations of cis 3-aminocyclohexanecarboxylic acid (ACHC 10-1000 µM, kindly provided by Dr. N.G. Bowery, St. Thomas' Hospital Medical School), β-alanine (10-1000 µM, Sigma) or (+) bicuculline (500 µM, Sigma). Six microCuries of (^{3}H) GABA in 100 µl of PSG were then added and aliquots were taken for analysis after a further 4 minutes.

Effects of osmotic shock on (^{3}H) GABA transport. One hundred microlitres of cell suspension (2-5 x 10^6 cells) were incubated with 1 ml of PSG containing 10 µl of (^{3}H) GABA. Six aliquots (100 µl) were taken for analysis after 4 minutes. Three aliquots were washed as already outlined. The remaining aliquots were treated in the same manner except that distilled water was substituted for 0.9 percent saline during washing.

Distribution of (^{3}H) GABA, (^{3}H) β-alanine and (^{3}H) glutamate uptake in enriched cell fractions. In these experiments the cells were labelled at an intermediate stage of the isolation, just before the final centrifugation through 4 percent BSA, and the cells were fixed in glutaraldehyde before separation. Ten millilitres of the crude cell suspension were incubated with 50 µCi of either (^{3}H) GABA, (^{3}H) glutamate or (^{3}H) β-alanine for 30 minutes. (^{3}H) GABA and (^{3}H) β-alanine incubations were performed in the presence of AOAA (10 µM final concentration) which was added to the cell suspension 15 minutes before the tritiated amino acids. (^{3}H) GABA incubations were also carried out in the presence or absence of ACHC (500 µM). The ACHC was added 4 minutes before the (^{3}H) GABA. Incubations were terminated by

centrifugation through 2 ml of 4 percent BSA in calcium and magnesium free Krebs Ringer buffer (CMF-KRB) (5 minutes, 100xg). The pellet was gently resuspended by trituration in 5 ml of CMF-KRB and the cells fixed by adding a further 5 ml of 5 percent glutaraldehyde in phosphate buffered saline. (Glutaraldehyde fixation was used to prevent the loss of label from the cells during the separation procedure, since it takes up to 4 hours to obtain enriched cell fractions from the cell suspension. In one experiment using (^{3}H) GABA the glutaraldehyde fixation step was omitted. After 15 minutes the cells were sedimented (5 minutes, 100xg) and resuspended in 10 ml of 0.2 percent BSA in CMF-KRB. Twenty microlitre aliquots of this fixed cell suspension were taken for cell counting. Coulter counter analysis showed no significant perturbation of cell size due to the fixation procedure. Up to 180 x 10^{6} cells were used in the cell separation (Cohen et al. 1978) which was performed as follows. The separation was carried out at 3^{o}C using solutions which had been gassed with 95 percent O_2 and 5 percent CO_2 after sterile filtering (0.22 µM Millipore membranes). The perspex separation chamber was of the type described by Miller & Phillips (1969). The chamber which was 12.5 cm in diameter was filled by upward displacement through a hole in the conical base with the following solutions in sequence: (i) 50 ml CMF-KRB (ii) 50 ml 0.2 percent BSA in CMF-KRB containing the fixed perikarya (iii) 500 ml of a discontinuous BSA gradient 0.5 percent -2 percent in CMF-KRB, generated from equal volumes of 0.5 percent and 2 percent BSA in CMF-KRB using an LKB gradient mixer. The perikarya were allowed to fall through the gradient for 2 hours. The chamber was emptied slowly (approximately 30 ml per minute) and around 50 x 10 ml fractions were collected for analysis using a fraction collector (Ultrorac, LKB). Alternate fractions were analysed for cell number and size distribution. The remaining fractions were analysed for radioactivity as outlined previously. The entire 10 ml fraction was filtered through glassfibre filters for analysis of radioactivity.

2.3 Results

Time course of (^{3}H) GABA and (^{3}H) glutamate uptake into cell perikarya. When the cell suspension was incubated with (^{3}H) GABA (0.17 µM) there was a rapid accumulation of label into the cells (Figure 5). Uptake at 25°C was linear for the first 4 minutes rising to 1.8 pmol/per 10^6 cells at 32 minutes. At this temperature AOAA did not have any significant effect. At 37°C (Figure 5 inset) uptake in the absence of AOAA was similar to that seen at the lower temperature. However in the presence of AOAA the apparent uptake at 37°C was significantly elevated after 32 minutes to 2.6 pmol per 10^6 cells ($t = 2.99$, $p<0.05$, 1-tailed paired Student's t-test). The initial rate of (^{3}H) GABA uptake was linear with cell concentrations up to 6 x 10^6 cells per ml (not shown).

(^{3}H) glutamate (0.16 µM) was also rapidly accumulated by the isolated perikarya at 25°C (Figure 6). The uptake was linear over the first 4 minutes rising to 1.15 pmol per 10^6 cells at 32 minutes. The initial rate was again found to be linear with respect to cell concentration.

Sodium dependence of (^{3}H) GABA transport. When the normal medium was replaced by 0.32 M sucrose, the uptake of (^{3}H) GABA was greatly reduced (Figure 7). The addition of 0.9 percent Nacl during the incubation resulted in a 13-fold increase in the rate of uptake.

Kinetics of (^{3}H) GABA and (^{3}H) glutamate transport. An Eadie-Hofstee plot of initial rate of (^{3}H) GABA transport and GABA concentration was found to fit a straight line (Figure 8, see also section 1.6). Thus the kinetics of (^{3}H) GABA transport are compatible with a high affinity carrier-mediated process with an observed Kt of 1.9 ± 0.2 µM and V of 1.8 ± pmol per 10^6 cells per minute. Similarly Figure 9 demonstrates that (^{3}H) glutamate uptake also proceeds by a high affinity transport process with the following kinetics Kt 10±2 µM and V 7.9 ± 0.8 pmol per 10^6 cells per minute.

Effect of inhibitors on (^{3}H) GABA uptake. Uptake of (^{3}H) GABA (0.1 μM) was inhibited by ACHC and β-alanine (Figure 10). ACHC was the more potent of the two with an IC_{50} of 70 μM (IC_{50}= concentration of inhibitor required to reduce uptake to 50 percent control). Although β-alanine did inhibit uptake, much higher concentrations were required (IC_{50}=600μM). Bicuculline has a small but significant effect at the high concentration of 500 μM reducing uptake to 80 control ($t=5.42, p<0.02$, 1-tailed paired Student's t-test, 2 degrees of freedom) (not shown).

Effect of osmotic shock on (^{3}H) GABA uptake. When the cells were washed with distilled instead of isotonic saline, after incubating with (^{3}H) GABA, the apparent uptake of this amino acid was significantly reduced to less than 9 percent of control ($t=19.5$, $p<0.001$, 1 tailed paired Student's t-test, 2 degrees of freedom) (not shown).

Transport of (^{3}H) GABA, (^{3}H) β-alanine and (^{3}H) glutamate into the enriched cell fractions. The greatest uptake of (^{3}H) GABA (0.6 pmol per 10^6 cells) was seen in E and D, which contained the largest cells (Figure 11a, Table 1). Uptake was lower (0.24 pmol per 10^6 cells) in the C fraction containing intermediate size cells, and was minimal in the B and C' fractions (0.06 pmol per 10^6 cells). In the presence of ACHC (500 μM), (^{3}H) GABA transport was reduced by 43% but there was no obvious change in the pattern of uptake. However the pattern of (^{3}H) glutamate uptake was notably different from that of (^{3}H) GABA (Figure 11b). In this case uptake into the D fraction was most prominent (1.37 pmol per 10^6 cells, respectively). Uptake was lowest in the B and C fractions. The distribution of (^{3}H) β-alanine (Figure 11c) resembled that of (^{3}H) glutamate in that uptake was greatest in the D fraction.

The A fraction contains a large proportion of subcellular particles smaller than the lower size limit for A (5.5 μM spherical diameter) and therefore estimates of cell numbers are innacurate. Nevertheless it appears

that there was significant transport of all three amino acids into this fraction.

In the experiment where the glutaraldehyde fixation step was omitted the amount of (^{3}H) GABA lost during the separation procedure was increased to 84 percent compared with 33 percent for fixed cells. However the pattern of uptake was the same whether or not fixation was carried out.

Since the cell fractions are of different sizes it is conceivable that the difference in uptake by the fractions could be a function of cell volume. However the finding that the patterns of uptake are different for the 3 amino acids indicates that this is not the case.

Figure 5 A summary of (^{3}H) GABA transport (GABA concentration = 0.17 μM) by the cell suspension. The time course (0-32 minutes) of uptake was examined at 25°C and 37°C (inset) in the presence (solid lines) and absence of AOAA (10 μM) (broken lines).

*At 37°C the apparent uptake of GABA was significantly increased by the presence of AOAA ($t = 2.9$, $p<0.05$, 1 tailed paired Student's t-test). The results are plotted as means ± sem; number of determinations in parentheses.

FIGURE 5

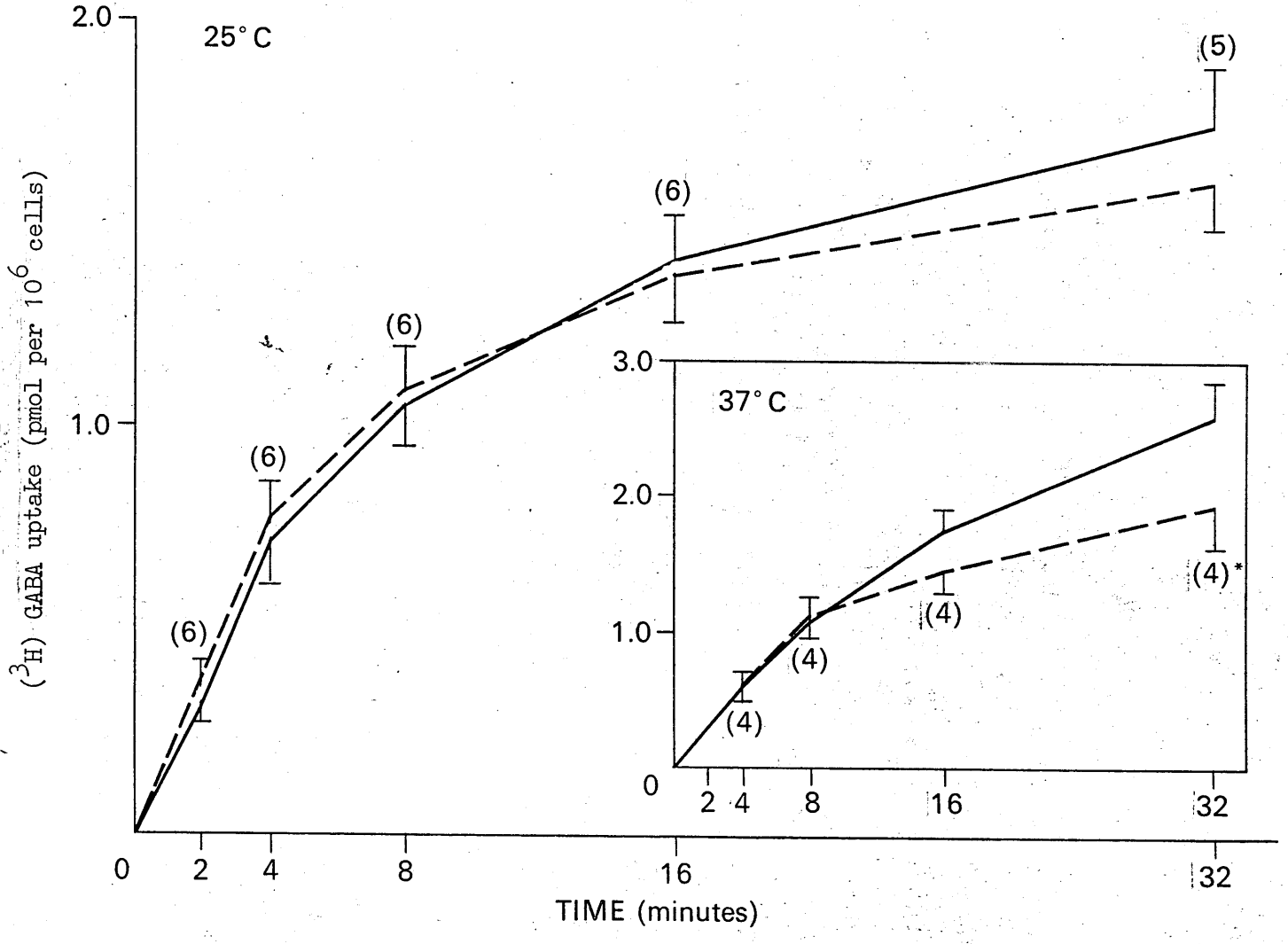

25° C
37° C
(3H) GABA uptake (pmol per 10^6 cells)
TIME (minutes)
2.0
1.0
0
3.0
2.0
1.0
0
2
4
8
16
32
(6)
(6)
(6)
(6)
(5)
(4)
(4)
(4)
(4)*

FIGURE 6 Transport of (^{3}H) glutamate (0.16 μM) by the cell suspension at 25°C over the time interval 0-32 minutes. Results of four determinations plotted as means ± S.E.M.

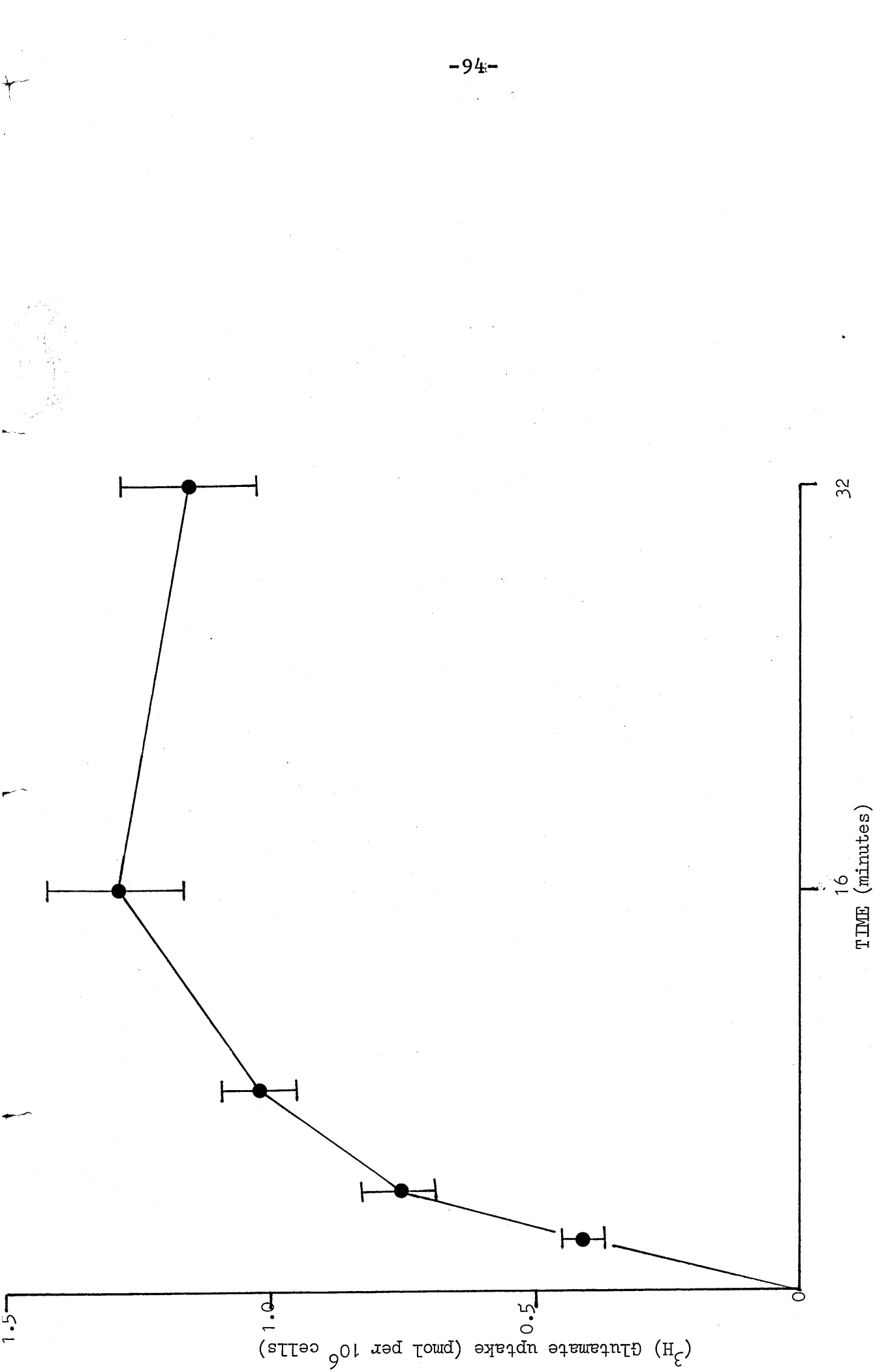

1.5
1.0
0.5
0
16
32
TIME (minutes)
(^{3}H) Glutamate uptake (pmol per 10^6 cells)

FIGURE 6

FIGURE 7 Na+ dependence of (^{3}H) GABA transport. Results of duplicate experiment plotted as means ± range. Incubations were initially in 0.32M sucrose. At 4 min. 0.9 per cent NaCl wasadded to give a Na+ concentration of 72mM.

FIGURE 7

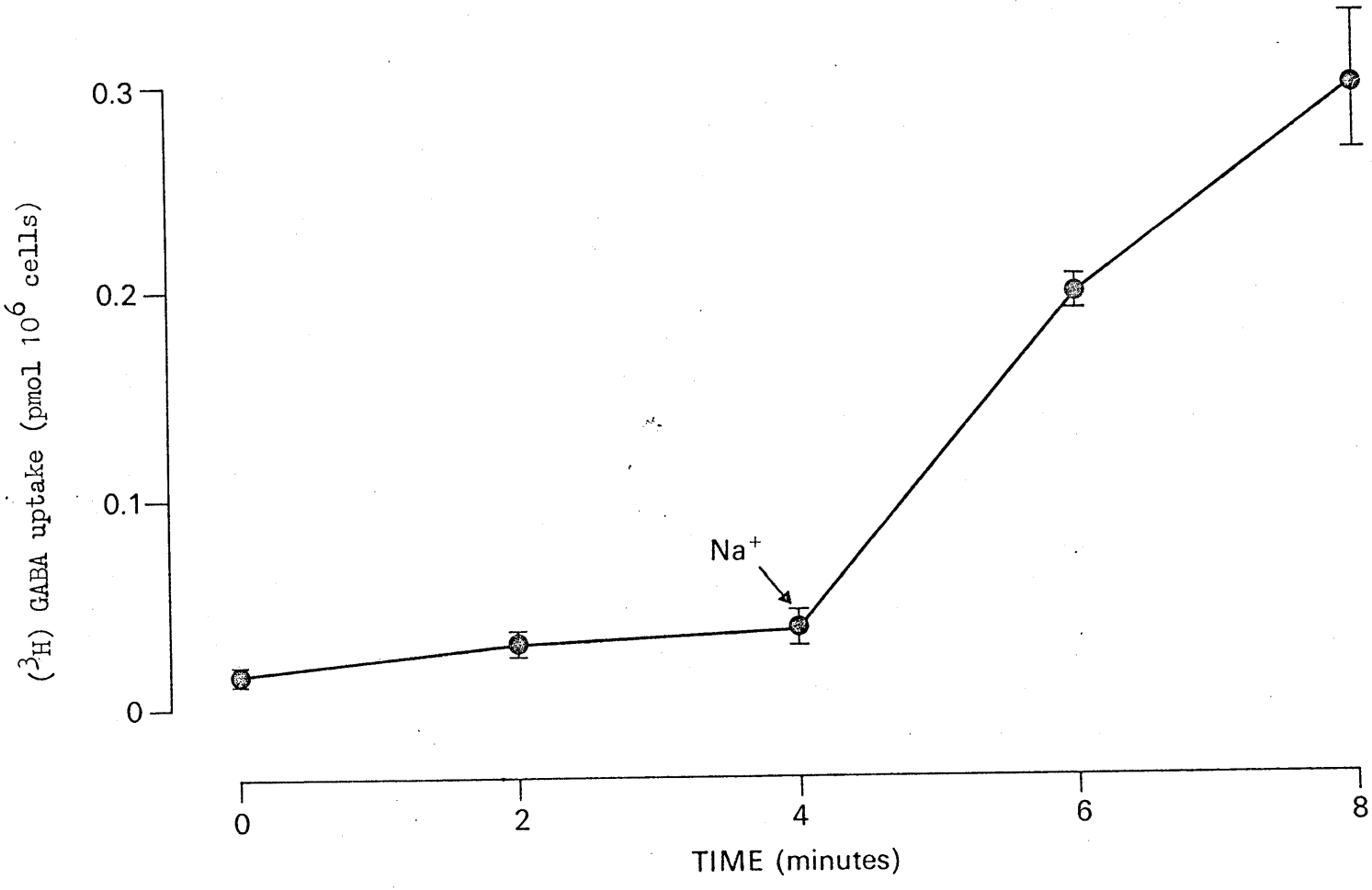


FIGURE 8 Eadie-Hofstee plot of (^{3}H) GABA transport. From this data V (1.8 ± 0.1 pmol per 10 cells per minute) and Kd (1.9 ± 0.2 μM) were calculated by linear regression (r = 0.92) (Edwards 1967). Results are from 2 experiments each data point representing one determination.

FIGURE 8

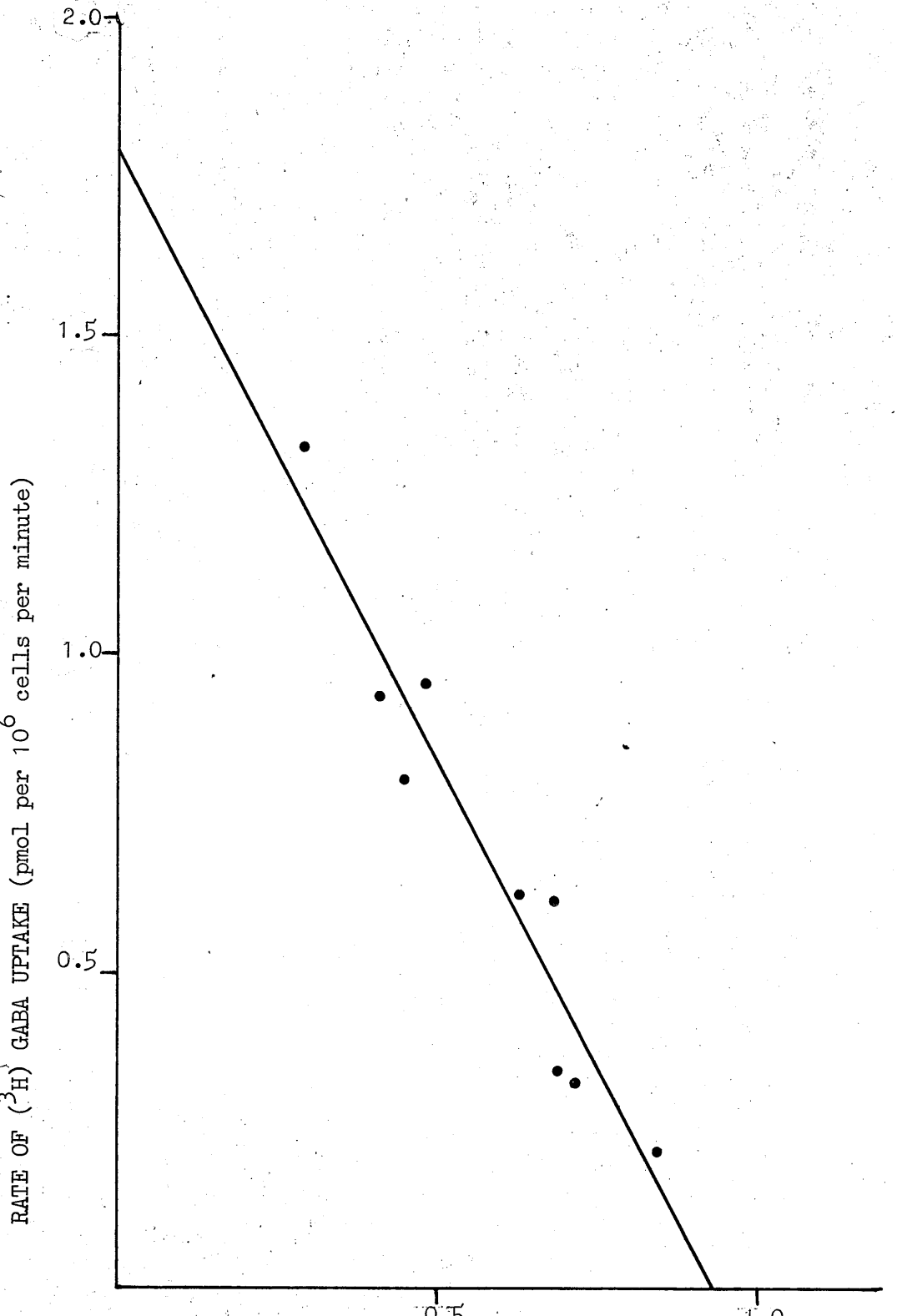

2.0
1.5
1.0
0.5
RATE OF (^{3}H) GABA UPTAKE (pmol per 10^6 cells per minute)
0.5
1.0
RATE OF (^{3}H) GABA UPTAKE (pmol per10^6 cells per minute per μM)
GABA CONCENTRATION

FIGURE 9 Eadie-Hofstee plot of (^{3}H) glutamate transport. From this data V (7.9 ± 0.8 pmol per 10^6 cells per minute) and Kd (10 ± 2 μM) were calculated by linear regression (r = 0.92) (Edwards, 1967). Each data point represents the mean of two determinations from duplicate experiments (range 18 per cent).

FIGURE 9

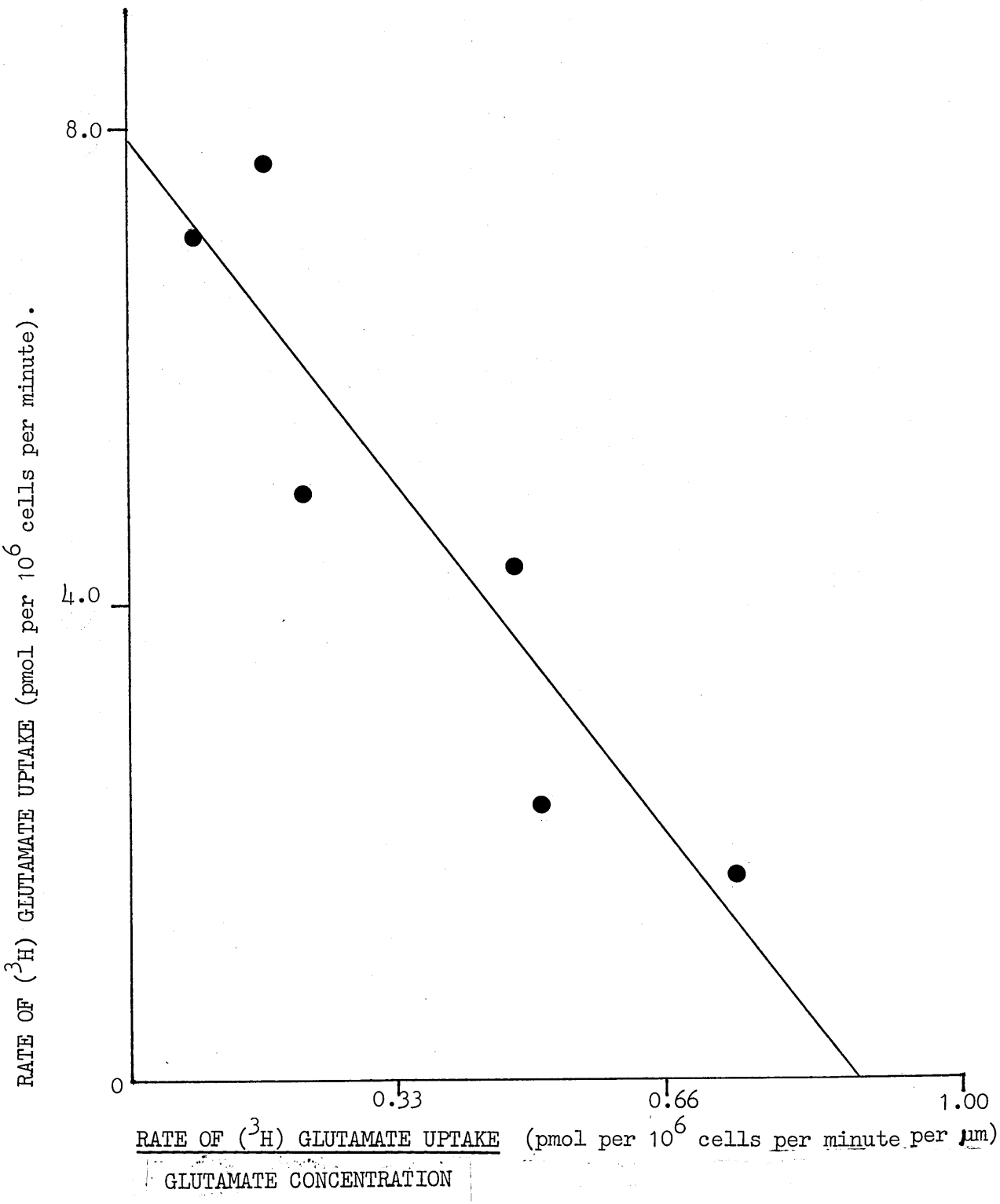
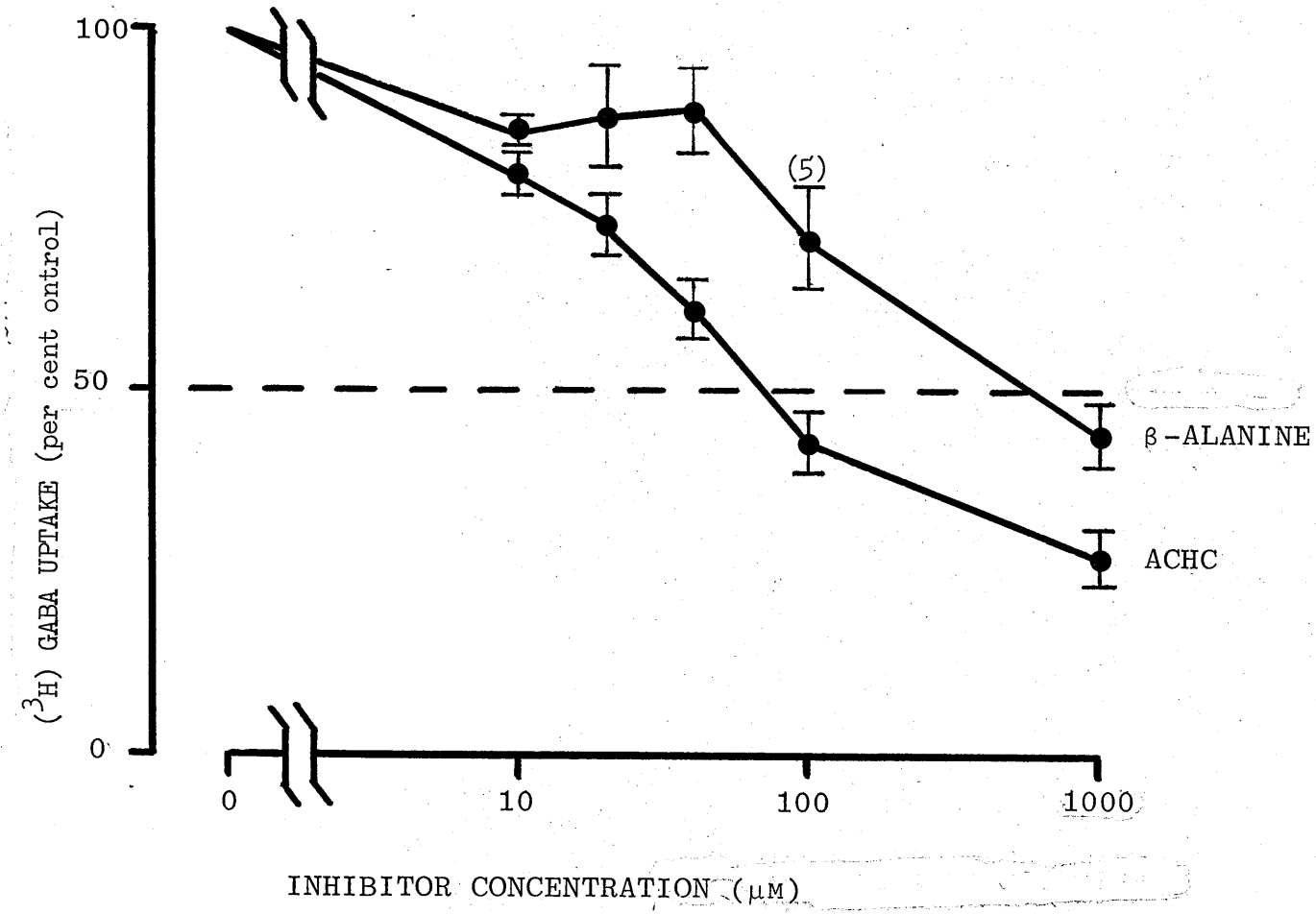

8.0
4.0
0
0.33
0.66
1.00
RATE OF (^{3}H) GLUTAMATE UPTAKE (pmol per 10^6 cells per minute).
RATE OF (^{3}H) GLUTAMATE UPTAKE (pmol per 10^6 cells per minute per µm)
GLUTAMATE CONCENTRATION

Figure 10 A summary of the effects of increasing concentrations of GABA transport inhibitors (ACHC and β-alanine) on the uptake of (^{3}H) GABA by the cell suspension. The IC_{50s} determined from this figure for ACHC and β-alanine were 70 μM and 600 μM respectively. The results are plotted as mean percent of control (i.e. percent of uptake in the absence of inhibitor) ± sem, against log inhibitor concentration (μM); n = 6 unless indicated otherwise in parentheses.

FIGURE 10


100
50
0
(5)
β-ALANINE
ACHC
0
10
100
1000
(3H) GABA UPTAKE (per cent ontrol)
INHIBITOR CONCENTRATION (μM)

FIGURES 11 a,b,c. Distribution of (^{3}H) GABA (± 500 μM ACHC) (^{3}H) β-alanine and (^{3}H) glutamate uptake in separated cell fractions. Results of single experiments are shown. All experiments were performed at least twice. Horizontal bars indicate predominant cell size in cell fraction (see Table 1).

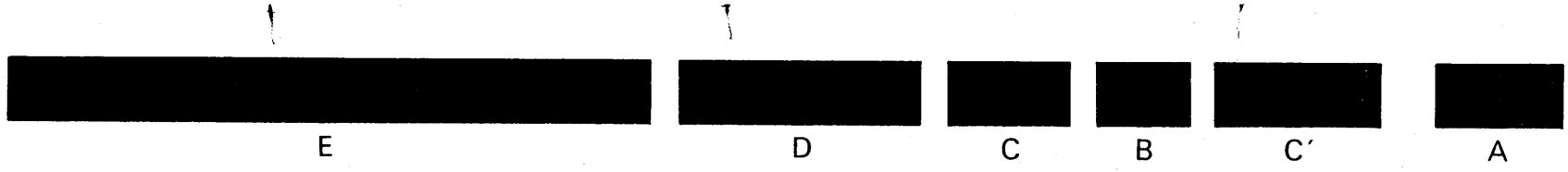


FIGURE 11a

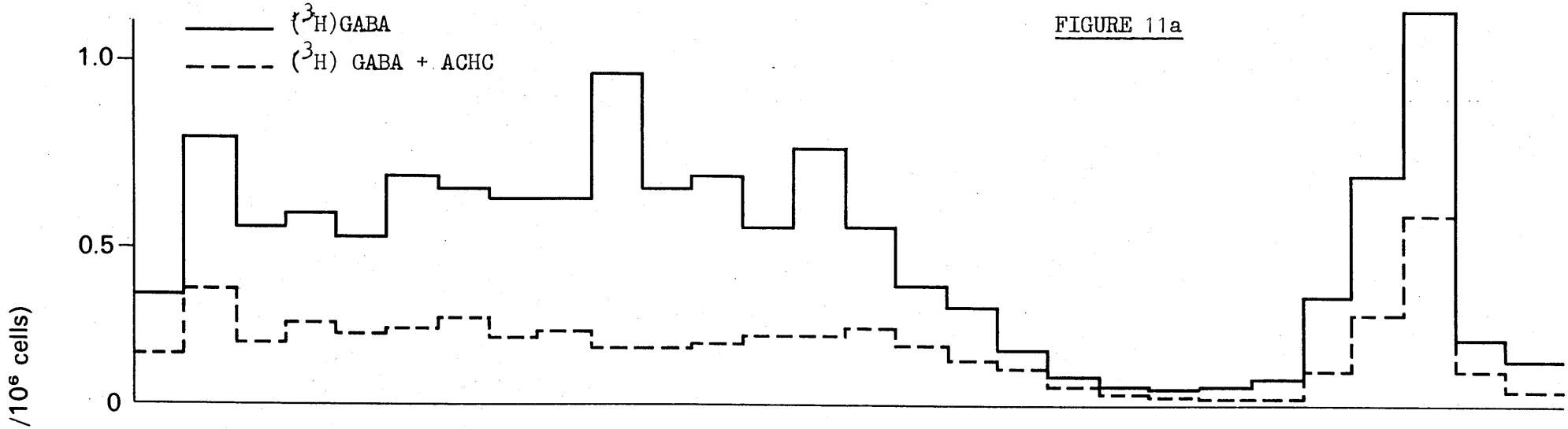


FIGURE 11b

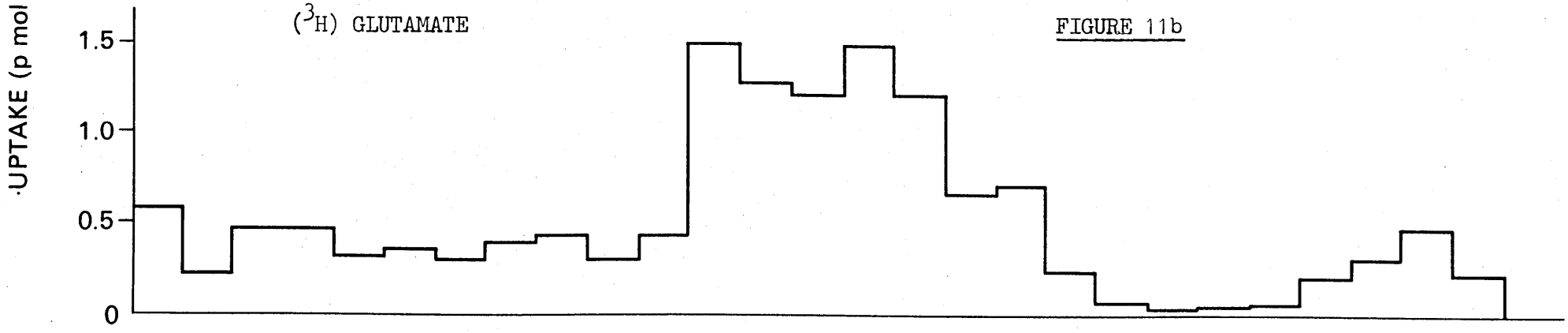
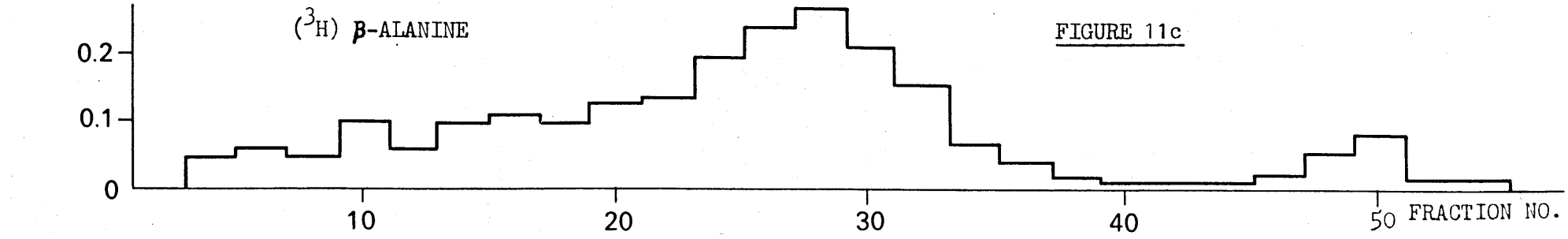


FIGURE 11c



UPTAKE (p mol /10^6 cells)

2.4 Discussion

The freshly prepared cell bodies from the developing rat cerebellum possess high affinity transport systems for GABA (Figure 8) and glutamate (Figure 9) with properties similar to those reported for various preparations of both the rat cerebral cortex and the cerebellum. High affinity uptake of GABA and glutamate has been demonstrated in slices (Iversen & Neal, 1968; Balcar & Johnston, 1972) and synaptosomes (Bond, 1973; Levi & Raiteri, 1973). Cultured cerebellar cells also possess a high affinity transport system for GABA (Lasher, 1975). In addition Hamberger (1971) has reported uptake of low concentrations of both GABA and glutamate into cells from the adult rabbit cerebral cortex produced by a sieving technique.

The Kt of 1.9 μM found here for GABA uptake from postnatal rat cerebellum compares with the values reported for various other preparations which range from 0.3 μM for cultured cerebellar neurons and glia (Lasher, 1975) to 22 μM for rat cerebral cortex slices (Iversen & Neal, 1968). Similarly the Kt for glutamate uptake (10 μM) is close to the values (20 μM) found for cerebral cortex slices and synaptosomes (Balcar & Johnston, 1972; Logan & Snyder, 1972). Accurate comparison of our findings on the maximal rate of transport (V) with the published values is difficult. However, since approximately 2.5×10^6 cells are equivalent to 1 mg wet weight of tissue, the maximal rates of transport of GABA and glutamate transport into the cerebellar total cell suspension approximate to 4.5 and 20 nmol per g wet weight per min.,respectively,and compare with 116 and 240 nmoles per g wet weight per min.for slices (Iversen & Neal,

1968; Balcar & Johnston, 1972). The slower rate of GABA uptake in our preparation may be partly explained by the loss of nerve terminals which are sheared off during the isolation procedure. A large number of high affinity transport sites for GABA are thought to be contained in the nerve terminals (Kelly & Dick, 1976; Iversen & Bloom, 1972). When one considers that the dendrites and axons have been estimated to account for 80-95 percent of the cell volume (Rose, 1972) then the large loss of uptake sites is not surprising. In addition trypsinisation is known to remove proteins from the cell surface (see Wilkin et al. 1976) and this may affect transport sites.

Several studies have reported that high affinity GABA transport requires the presence of extracellular sodium (see Section 1.6) (Weinstein et al.1965; Iversen & Neal, 1968; Lasler, 1975; Schousboe et al. 1977a). In this study sucrose was substituted for sodium chloride, therefore the possibility that chloride ions are responsible for the reduction in transport seen in the sucrose medium cannot be ruled out. However previous workers (Weinstein et al. 1965; Iversen & Neal, 1968; Lasher, 1975; Schousboe et al. 1977a) have shown that chloride ions are not directly implicated in the transport of GABA. Thus it seems likely that the reduction in sodium ions accounts for the deficit in GABA transport in the sucrose medium. It should perhaps be noted that on adding the sodium chloride to the sucrose medium, the concentration of sodium ions was increased to only 50 percent of that found in PSG. This may partly explain why the rate of GABA transport is still reduced when compared with that found for cells in PSG.

The high affinity uptake of amino acids reported and cited in this chapter can be distinguished from the high affinity binding of amino acids and other compounds outlined in Chapter 3, in a number of ways. Iversen and Neal (1968) reported that after incubating rat cerebral cortex slides with (^{3}H)GABA the concentration of label in the slices rose to 100-fold of that in the medium. Thus GABA was presumably being transported against a concentration gradient by an active process. This was confirmed by the finding that

metabolic inhibitors and ouabain (which inhibits sodium dependent active transport indirectly by blocking Na/K-ATPase) severely reduced GABA transport (Iversen & Neal, 1968; Lasher, 1975). Similar findings have been reported for glutamate (Balcar & Johnston, 1972; Logan & Snyder, 1972). However the tissue to medium ratios achieved for glutamate were around 40:1 (Balcar & Johnston (1972). It is possible to estimate the tissue to medium ratios for freshly prepared cells from the data in Figures 5 and 6 using the cells per mg wet wt approximation discussed on page 103. The tissue to medium ratio for both GABA and glutamate approximate to 20:1 after 30 minutes. This strongly suggests that these amino acids are being transported into cells. This somewhat lower figure for GABA, glutamate transport into freshly prepared cells compared with slices (Iversen & Neal, 1968; Balcar & Johnston, 1972) may be due to the leakiness of these disrupted cells and or a function of the slower rate of transport. Further evidence for the internal accumulation of GABA comes from the finding that when these cells are lysed with distilled water, GABA is lost from the cells. This loss of GABA would not have been expected if GABA were bound to the surface of these cells.

The use of neuronal and glial inhibitors of GABA uptake (ACHC and β-alanine, respectively) indicate that over the short time interval studied (4 minutes) most of the uptake is into neurons since ACHC is the more effective inhibitor. The IC_{50} found for ACHC is 70 μM which compares well with the value reported for rat cerebral cortex slices (62 μM) where the uptake is almost totally neuronal (Neal & Bowery, 1977). However, some GABA transport remains even in the presence of 1mM ACHC suggesting that there is some transport of GABA by glia. This is further supported by the finding that GABA transport is inhibited by low concentrations (10-100 μM) of β-alanine.

The predominance of neuronal (^{3}H) GABA transport contrasts with the observations of Hamberger (1971) on bulk prepared cells from adult cerebral cortex where uptake of (^{3}H) GABA was mainly into the fraction

designated as "glial". At 1mM concentrations of β-alanine and ACHC there appears to be some overlap in the specificity of these inhibitors since their combined inhibition exceeds 100 percent. This may be explained by the observations of Neal & Bowery (1977) and Bowery et al. (1976) which suggest that both inhibitors are capable of increasing the release of endogenous GABA perhaps sufficiently to reduce the transport of (^{3}H) GABA. AOAA has relatively little effect on GABA transport at 25 and 37^{o}C (except after 32 minutes at 37^{o}C) suggesting also that uptake of GABA is mainly into neurons since AOAA is known to greatly elevate the apparent uptake of (^{3}H) GABA into glia (Schon & Kelly, 1974b) but not over short time intervals, into neurons (Snodgrass & Iversen, 1973). Bicuculline, which inhibits the post-synaptic actions of GABA but not its transport (see Sections 1.6 and 1.7) is capable of effecting only 20 percent inhibition at the high concentration of 500 μM confirming that binding to the post-synaptic receptor does not play a significant part in these studies.

The metabolism of (^{3}H) GABA and (^{3}H) glutamate has not been examined in these studies. The metabolism of GABA is unlikely to have been important for the following reasons. With the exception of one experiment all the studies were performed at 25^{o}C. Figure 5 shows that at this temperature, AOAA, which blocks GABA transaminase, the enzyme responsible for GABA metabolism (Snodgrass & Iversen, 1973) has no significant effect on the apparent uptake of GABA by the cells. Thus it seems unlikely that significant amounts of GABA were metabolised during the first 30 minutes of incubation. Glutamate metabolism may have been more of a problem. Balcar & Johnston (1972) showed that in rat brain slices, incubated under similar conditions to those used here, over 90 percent of (^{3}H) glutamate remained unmetabolised after 8 minutes. Thus it seems probable that even over the 30 minute incubation period used to study glutamate uptake into the enriched cell fractions, the majority of the glutamate would be unmetabolised.

The distribution of (^{3}H)GABA, (^{3}H)glutamate and (^{3}H) β-alanine (Figure 11) in enriched cell fractions shows some interesting differences. (^{3}H) GABA which is known to be transported into the GABAergic Purkinje and Golgi neurons both in vivo (Schon & Iversen, 1972; Hökfelt & Ljungdahl, 1972) and in vitro (Hösli & Hösli, 1976; Ljungdahl et al. 1973; Lasher, 1974) is taken up mainly into the E and D fractions. This uptake is inhibited by ACHC which is consistent with an enrichment of larger inhibitory neurons in these two fractions (see Table 1). β-alanine which is almost exclusively accumulated by glia (Schon & Kelly, 1975; Kelly & Dick, 1976), is taken up mainly by the D fraction indicating an enrichment of glial cells. (^{3}H) glutamate is also accumulated mainly by the D fraction. Since a number of workers have reported the uptake of (^{3}H) glutamate into glia (McLennan,1976; White & Neal, 1976; Henn et al. 1974) it is probable that uptake of (^{3}H) glutamate into the D fraction is mainly into this cell type. However, uptake of (^{3}H) glutamate is often cited as a property of glutamatergic cells (Young et al. 1974; Storm-Mathisen, 1977; Iversen & Mathisen, 1976). Since the granule cells are thought to use glutamate as their neurotransmitter (Young et al. 1974; Hudson et al. 1976; Sandoval & Cotman, 1978), one might expect to see a peak of glutamate uptake in cell fractions enriched in granule cells, i.e. the C and B fractions. However, uptake into these two fractions is low compared with the D fraction. It is possible that glutamate uptake may occur mainly into granule cell dendrites (which are removed during isolation) and not into the perikarya. Such dendrites would be found in the A fraction. However glutamate uptake into this fraction is relatively low and could be accounted for by the presence of glial fragments since similar uptake is seen for β-alanine. The large amount of GABA uptake seen in the A fraction indicates that dendrites of GABAergic neurons may occur in this fraction. The results for glutamate transport compare well with the findings of Campbell & Shank (1978) who report that transport is three times more rapid into glia than into granule enriched populations. Unfortunately their data are expressed in uptake per

mg protein, and accurate comparisons are difficult. Campbell & Shank (1978) came to the conclusion that uptake of glutamate in the granule cell fraction was not the result of glial contamination for the following reason. The ratio of GABA: glutamate uptake was much greater in the glial fraction compared with the granule cell fraction, whereas one would predict that the ratios should be equal if glia were responsible for GABA and glutamate uptake in the two fractions. However as Campbell & Williams (1978) point out, their glial fraction contains some neurons (Purkinje and interneurons) which might account for the rapid uptake of GABA in this fraction. A comparison using β-alanine instead of GABA might have been more revealing. Messer (1977) reported that it was not possible to show specific accumulation of (^{3}H) glutamate in cultures from the developing mouse cerebellum and studies of rat cultures prepared in this lab confirm this (Pearce, Currie & Dutton, unpublished).

It has been previously reported that the perikarya isolated from the developing rat cerebellum are metabolically active (Wilkin et al. 1976) and can be cultured (Currie et al.1979). In this study it has been demonstrated that these cells retain high affinity transport systems for both GABA and glutamate which compare with those in intact tissues. Using the unit gravity sedimentation technique the uptake of these tritiated amino acids gives further information about the identity of the cell fractions and provides added evidence that the uptake of (^{3}H) glutamate in the cerebellum is primarily into glial cells and not the presumed glutamatergic granule cells.

2.5 Future directions

Perhaps one of the most obvious experiments to do is to characterise GABA transport into neurons and glia using the unit gravity separation technique, since to my knowledge no one has examined the kinetics of GABA transport in neurons and glia freshly prepared from the same brain region. Unfortunately as can be seen from Figure 11 and Table 1, the glial enriched fraction between fractions D and C is contaminated by interneurons. However Cohen et al. (1980), using similar techniques for the isolation and separation of cerebellar neurons, avoided this problem by depleting the interneuron population. This was achieved by the administration of the anti mitotic hydroxyurea (Ebels et al. 1975) to 6 day rats, thus preventing the formation of interneurons and granule cells from their precursors. This treatment also allows greater numbers of Purkinje and Golgi neurons to be separated since 180 x 10^6 cells is maximum number of cells that can be separated on the sedimentation chamber and usually most of these cells are granule neurons. Using this technique relatively pure populations of GABAergic neurons (Purkinje and Golgi cells in the E fraction) and glial cells (mainly astrocytes in the latter part of the D fraction) (see also Table 1) were obtained. The kinetics for GABA transport into these fractions was similar to that reported in this chapter, but the Kt for neuronal transport (approx. 2uM) was twice that found in this study for glia. Similarly, the maximal rate of GABA transport into neurons (0.310 nmol per 10^6 cells per min.) was 6-fold higher than that for glial cells. The faster rate of GABA transport into neurons is in accord with the conclusions drawn from

GABA uptake into neurons and glia from different regions of the nervous system (see Section 1.6). It should perhaps be noted that the values for maximal transport of GABA by neurons reported by Cohen et al. (1980) were about 150 fold nigher than reported in this Chapter for the cell suspension. This may be explained by the presence of large numbers of granule cells, which do not contribute significantly to GABA transport (see Figure 11) in the cell suspension (the E cells account for about 1 per cent of cells in the cell suspension, Table 1). These workers also confirmed the specificity of ACHC and β-alanine in inhibitory neuronal and glial GABA transport.

As mentioned in Section 1.5 methods have been developed in this lab for the culture of cerebellar neurons and when this work was initiated it was hoped that the GABA transport would be extended to the cerebellar cultures. Since that time a significant amount of autoradiographic data have been accumulated in this lab concerning the uptake of (^{3}H) GABA and (^{3}H) glutamate and the specificity of the inhibitors ACHC and β-alanine all of which essentially support the findings of this Chapter (Currie & Dutton, 1978; Pearce & Dutton, unpublished). The ultimate aim of such studies is the identification of cell types in culture, i.e. ACHC-sensitive GABA transport characterises neurons and β-alanine sensitive uptake characterises glia.

CHAPTER 3

(^{3}H) MUSCIMOL ^{3}H DHA AND (^{3}H) QNB BINDING SITES IN NORMAL AND NEUROLOGICALLY MUTANT MICE

3.1 Introduction

In Sections 1.2 and 1.3 the development of the cerebellum in normal and neurologically mutant mice (wv, rl, sg and jp) has been outlined and the neuropathology of these mutants discussed. In this chapter a study of the binding of neurotransmitter receptor ligands (^{3}H) muscimol, (^{3}H) DHA and (^{3}H) QNB to the total particulate fractions of developing normal and neurologically mutant mice is outlined. These ligands allow the characterisation of GABA, β-adrenergic and muscarinic receptors (see section 1.7). (^{3}H) DHA and (^{3}H) muscimol were chosen for the study because β-adrenergic and GABA receptors are known to be involved in the functioning of the cerebellum and there is information regarding the location of these receptors (see section 1.4). (^{3}H) QNB was included since although the function and precise location of these receptors in the cerebellum is not known (Rotter et al. 1979b), it was hoped that the following study might provide answers to these questions.

The aims of the study were as follows:

(i) to gain some idea of the time course of development of receptors, and to compare this with what was known about the synaptogenic events occurring in the cerebellum.

(ii) to use the mutants to examine receptor development in the absence of granule cells in order to gain information concerning, the function of these neurons, and the way in which the reorganisation of the cerebellum following granule cell loss affects receptor development.

The possibility of performing binding studies on cell enriched fractions was considered. However there are a number of difficulties involved in this sort of work. For example suspensions of cells can be obtained only from mice aged 8 days or younger. At this stage of development, the majority of DHA, muscimol and QNB binding sites have not been formed, although the cerebellum does contain significant numbers of these receptors at 8 days. Another problem is that as outlined in Chapter 2, the dendrites, the main sites of synaptic contact are removed by the isolation procedure. Thus one might expect the major sites of receptors to be lost. However perhaps the largest drawback is the extremely small amount of material, which is available from the fractions, on which to perform analyses. The pooled E and D fractions contain less than 100 µg of protein each. Thus it would be extremely difficult to obtain sufficient material to obtain an accurate estimate of the concentration of binding sites on E and D cells. (About 2.5 mg protein, was used to assay muscimol receptors in mutant animals). Since the remaining fractions contain granule cells, the exercise would be unlikely to yield any information concerning the cellular location of neurotransmitter binding sites. However it is hoped that an alternative strategy may be adopted to answer such questions (see Section 3.5).

3.2 Materials and methods

Breeding of normal and neurologically mutant mice. Unless stated otherwise, all mice used in the following studies were produced in this institution by sibling crosses. These animals originated from stock imported from the Jackson Laboratory (Bar Harbor, U.S.A.). For binding studies in normal mice animals of the B6/CBA hybrid strain (of either sex) were used throughout. However adult male CBA animals (age 6-10 weeks) supplied by Olac (Bicester) were used in the cerebellar glomerulus studies. The following procedures were adopted for the breeding and identification of neurological mutants.

Weaver. These mutants (background strain B6/CBA) were produced by the crossing of heterozygous wv siblings from litters which had previously produced mutants. Mutants were identified by their uncoordinated gait which was easily recognisable in animals older than 8 days. Heterozygous wv cannot be identified behaviourally, and thus not all pairs selected for breeding produced mutants. (The probability of selecting two heterozygous wv from a mutant producing litter is 0.40. In the colony of 48 breeding pairs used in these studies about 50 percent of these matings were eventually rejected as non-mutant producers.

Reeler In the (^{3}H) QNB binding study rls of the C57BL/6J strain were used. The mutants were produced as outlined above for wv by crossing heterozygous rls which were identified by their unsteady gait (the symptoms are first apparent around 10 days). The heterozygous rls cannot be identified, and thus, as with the wv mutants, not all breeding pairs produce mutants. C57BL/6J rl colony consisted of 48 breeding pairs, however this mutation was transferred to the B6/CBA background to bring it in line with the wv and jp mutations. In addition, previous

studies have shown that rl mutants maintained on hybrid background strains were more robust than their inbred counterparts (Caviness et al. 1972). The transfer of the rl mutation from the C57BL/6J to the B6/CBA background was carried out as follows: C57BL/6J rl carrying females (identified by their breeding record) were mated with CBA males (supplied by Olac) to produce the hybrid B6/CBA. The rl carrying offspring of this mating were then identified by back-crossing with proven (C57BL/6J carriers). The rl carrying hybrids thus identified were inbred for at least seven generations before use. The rationale for the production and identification of these hybrid rl was identical to that outlined for the C57BL/6J animals.

Staggerer. These mutants were of the C57BL/6J strain. The problems associated with the selection of heterozygous rls and wvs for the production of mutants was almost eliminated by the use of the marker genes dilute (d) and short ear (se). These recessive genes lie close to the sg locus and in the colony used here they were associated with the unaffected chromosome. Thus normal (non-sg carrying animals) express these genes, i.e. they have grey coats and short ears. Heterozygous animals for breeding purposes were identified by their dark coats and lack of neurological symptoms. Sgs were identified by their ataxia which was apparent around 8 days. This classification of animals on the basis of marker genes does not take into account the phenomenon of recombination (i.e. crossovers, where a gene is transferred between chromosome pairs during meiosis). However the two marker genes are relatively close to the sg locus, thus recombinations are uncommon (approximately 5 percent).

Jimpy. This gene is sex linked and is expressed by male animals carrying the gene. These mutant males were identified by their characteristic shaky gait as early as 10 days. The mutants are produced by crossing normal males and carrier females. Thus half the male offspring will be jp mutants. In order to facilitate the identification of female jp carriers the mutation was linked with the dominant coat marker tabby (Ta). Thus carriers have a tabby coat as do most jp males. However the loci for these genes are relatively far apart and 10-20 percent recombination takes place. Thus, 1 in 5 to 1 in 10 tabby females do not carry the jp gene and the same proportion of tabby males are not mutants.

Control animals. Controls for the studies involving the mutant animals were taken from non-mutant producing colonies or in the case of sg non-carrier animals identified by their short ears and grey coat were used. Where possible control animals were of the same background strain as their mutant counterparts. However in the (^{3}H) QNB binding study C57BL/6J controls were not available and a closely related strain C57BL/10 were used. No attempt was made to limit the litter size of either normal or mutant litters since mutants were not readily identifiable until the second postnatal week. However animals from small (less than 4) or large litters (greater than 10) were not used. In selecting cerebella for analysis each determination was performed on material from the litters of one or more breeding pairs and no more than two determinations were carried out on material from the same source. Thus the material for five determinations was obtained from the litters of at least three separate breeding pairs.

Preparation of cerebellar glomeruli. These particles were made by a modification of the method described by Hajos et al. (1975). The cerebella of 12 male CBA mice (aged 6-10 weeks) were removed and cleared of meninges as described in Section 2.2. The cerebella were chopped at 1 mm intervals in two directions (the second pass being at right angles to the first). Hereafter all procedures were carried out at 0-4°C and all solutions contained 1 mM magnesium sulphate. The chopped cerebella were transferred to a modified Dounce homogeniser, containing 8-10 ml of 0.3 M sucrose, and homogenised by 30-35 strokes of a stainless steel pestle (radial clearance 0.25 mm). This pestle was then replaced by another of narrower clearance (0.12 mm) and the tissue further homogenised by 50-55 strokes. The homogenate was transferred to a beaker and the homogeniser washed out with a further 3-4 ml of sucrose solution. The homogenate was filtered under gentle pressure through nylon and metal mesh filters (pore sizes 160 μm and 40 μm, respectively) (Stanier, Manchester) inserted on to the end of a cylinder prepared by removing the end of a 10 ml syringe barrel. Five hundred to one thousand microlitres of this filtered homogenate were retained for analysis and the remainder was centrifuged (10 minutes, 900 xg). The supernatant was retained for analysis and the pellet gently resuspended in isotonic sucrose (final volume 10 ml) by trituration using a 1 ml Eppendorf pipette. The suspension was centrifuged again and the procedure repeated. The final pellet was resuspended in 6 ml of 0.3 M sucrose. Three portions of approximately 2 ml were then layered on a linear gradient (in a 23 ml polycarbonate centrifuge tube (MSE) generated from 7.5 ml of 1.2 M and 1.35 M sucrose solutions. This gradient was under layered with a cushion of 5 ml of 1.4 M sucrose. These tubes were centrifuged (3 x 25 ml (MSE 43127-104) (60 minutes, 53,000 x g)) after which 4 ml and 3 x 6 ml aliquots, were drawn off from the bottom of the tube, in that order. The glomeruli were concentrated at the gradient cushion

interface and were thus taken up in the second aliquot. The four fractions thus obtained were diluted with distilled water to give a final volume of around 40 ml. The diluted fractions, the original homogenate and the first supernatant were centrifuged (30 minutes, 50,000 x g) and the pellets resuspended in 2 ml of buffer (appropriate to the binding study for which these fractions were to be used). This material was stored at -20°C to await analysis.

(^{3}H) Muscimol binding. A modification of the method outlined by Snodgrass (1978) was used to study the binding of this ligand. Cerebellar tissue (25-55 wet wt.) was homogenised using a Polytron homogeniser (Kinematica) in 2 ml of ice cold distilled water. Homogenates were centrifuged (50,000 x g for 20 minutes at 3°C). Pellets were resuspended in 2 ml distilled water and stored at -20°C for at least 24 hours before analysis. (Freezing the tissue had the effect of increasing specific binding by 35% (not shown; $t = 3.29$, $p<0.025$, Student's t-test). Prior to analysis homogenates were made up to 7 ml with ice cold distilled water and centrifuged (50,000 x g for 20 minutes at 0°). Pellets were resuspended in a further 7 ml of cold water, centrifuged, and the final pellets resuspended in 2.2 ml of ice cold water. This procedure was also adopted for cerebellar glomerular material. Duplicate 500 µl aliquots of this suspension were incubated at 0°C for 10 minutes in 5 ml polypropylene centrifuge tubes (Sarstedt) with 1 ml of 60 mM HEPES-Tris buffer (pH 6.8) containing (N-methylamine-^{2}H) muscimol ((^{3}H) muscimol, 19 Ci per mmol, Radiochemical Centre, Amersham) in the presence or absence of 100 µM GABA (Sigma). Specific (^{3}H) muscimol binding reached equilibrium very rapidly, most of the binding had occurred within 1 minute and was complete by 10 minutes (Figure 12a). Incubations were terminated by centrifugation at 50,000 xg for 10 minutes at 0°C. The supernatants were aspirated and the pellets superficially washed twice with 5 ml of

ice cold water. The pellets were solubilised by heating to 50°C for 1 hour with 100 µl of 0.1M NaOH. The solubilised pellets were transferred to scintillation vials and the radioactivity was measured by liquid scintillation specroscopy using 10 ml of 0.6 percent PPO (Sigma) in 4:1 toluene: methoxyethanol. Alternatively the samples were counted using 8 ml of the scintillant "Cocktail T" (Hopkin and Williams) plus 1 ml of distilled water. Counting efficiency ranged from 26-34 percent and correction was made for quenching by the external standards ratio technique (Peng 1977). The apparent dissociation constant (Kd) for (^{3}H) muscimol binding was determined using a range of (^{3}H) muscimol concentrations from 0.5 - 33 nM. In all other experiments the (^{3}H) muscimol concentration was fixed at 2.8 nM. Specific binding was taken to be that displaced by 100 µM GABA and this binding varied from 80 percent of total counts for binding to tissue from normal animals to 30 percent for binding to mutant tissue. Binding was linear with protein concentrations up to 0.55 mg protein per incubation (this limit was not exceeded) and was maximally displaced by 100 µM GABA (IC_{50} ≃ 130 µM). Protein concentrations were determined by the method of Lowry et al. (1951).

(^{3}H) DHA binding. This was determined using a modification of the method described by Bylund (1978). Cerebellar tissue (25-55 mg wet wt.) was homogenised using a Polytron disintegrator in 2 ml of ice cold 0.05 M tris HCl buffer (pH 8.0 at 25°C). The homogenate was stored at -20°C to await analysis performed as follows. (Freezing the tissue had no significant effect on binding). Homogenates were made up to 7 mls with buffer, centrifuged (50,000 xg, 20 minutes, 3°C). Pellets were resuspended in a further 7 ml of buffer and again centrifuged. The final pellets were resuspended in 4.1 ml of buffer for assay. Duplicate 900 ml aliquots of the homogenates were incubated at 25°C for 25 minutes with 100 µl of buffer containing 1 (propyl-2, 3-^{3}H)

dihydroalprenolol ((^3H) DHA), 32-59 Ci per mmol (Radiochemical Centre, Amersham) in the presence or absence of 1 µM l-isoproterenol bitartrate (isoprenalin (Sigma)). (Specific (^3H) DHA binding reached equilibrium within 15 minutes and remained constant for at least 25 minutes (Figure 15a)). Incubations were terminated by the addition of 5 ml of ice cold buffer and rapid filtration through glass fibre filters. The filters were immediately washed twice with a further 5 ml of cold buffer. Radioactivity on these filters was determined using the method outlined **previously.** The Kd of (^3H) DHA binding was determined using a range of (^3H) DHA concentrations from 0.1 to 2.2 nM. In all other experiments binding was performed using a fixed concentration of (^3H) DHA (0.5 nM). Specific binding was taken to be that displaced by isoproteronal. This binding varied from 50 percent of total counts for binding to tissue from normal animals, to 15 percent for binding to mutant tissue. Binding was linear with cerebellar tissue concentrations up to mg protein per incubation (this limit was not exceeded). Specific (^3H) DHA binding was displaced by 97 percent by 1 µM isoproteronol ($IC_{50} \simeq 30\mu M$).

(^3H) QNB binding. The binding of this ligand was examined using a modification of the method described by Yamamura & Snyder (1974). Cerebella were homogenised using a Polytron disintegrator or glass-teflon mortar and pestle in 0.05 M sodium potassium phosphate buffer (pH 7.4) (final tissue concentration approximately 5-15 mg per ml). When the cerebral cortex was examined the tissue concentration was reduced to 3 mg per ml to reduce the level of specific binding to that of the cerebellum. The protein concentration for material from the cerebellar glomeruli preparation was 0.5 - 2.7 mg per ml. Triplicate 100 µl aliquots of these homogenates were incubated with 1 ml of buffer containing (3-^3H) Quinuclidinyl-benzilate ((^3H) QNB, 8-16 Ci per mmol, Radiochemical Centre, Amersham) in the presence or absence of 10 µM (Sigma) for 60 minutes at 25°C. (Specific (^3H) QNB binding reached equilibrium within 20 minutes and thereafter remained constant for at least 60 minutes (Figure 18a)).

The incubations were terminated by the addition of 5 ml of ice cold buffer and rapid filtration under suction through glass fibre filters (Whatman, GF/C). The filters were immediately washed 3 times with 5-10 ml of ice cold buffer. The radioactivity on the filters was then counted as outlined for (^{3}H) muscimol binding. The Kd of (^{3}H) QNB binding was determined using a range of (^{3}H) QNB concentrations (0.04-2 nM). All other studies were performed using a fixed QNB concentration (2nM) which gives greater than 90 percent saturation of the QNB receptors. (Although QNB binding was not examined at concentrations in excess of 2nM the use of this concentration was felt to be justified for the following reason. A number of workers have examined (^{3}H) QNB binding in excess of 2nM and lower affinity sites were not revealed (Yamamura & Snyder, 1974a; Sugiyama et al. 1977; Mallol et al. 1979)). Specific binding was taken to be that displaced by atropine, i.e. the difference between binding in the presence and absence of atropine (typically 75 percent of total binding). Saturation binding was linear with cerebellar tissue concentrations up to 2.4 mg wet wt. per incubation and was maximally displaced by 10 μM atropine (IC_{50} 8 nM).

3.3 Results

(^{3}H) Muscimol binding. Examination of the kinetics of (^{3}H) muscimol binding by Scatchard Analysis (Figure 12) indicated that this ligand was bound with high affinity to a single population of receptors (Kd = 13 $\pm$ 1 nM, B_{max} 1270 $\pm$ 100 pmol per g protein at 20 days). A statistical analysis of the data in Figure 12 (Edwards, 1967) showed that the Kds at 10 and 20 days were not significantly different (Figure 12, legend). Although significant levels of binding were found at the earliest age examined (0.028 pmol per cerebellum, at 5 days) development of (^{3}H) muscimol binding occurred mainly postnatally (Figure 13). The maximum increase in binding occurred between 15 and 20 days and binding levels continued to increase up to 30 days.

Although there was no significant increase in binding after 25 days there was a slight but significant reduction in binding between 30 and 40 days ($t = 3.07$, $p<0.02$, 2-tailed Student's t-test). With the exception of jp, (^{3}H) muscimol binding was reduced in all mutant cerebella (Figure 14, see also Table 3 for a summary of the analysis of variance of this data). Binding per cerebellum was most reduced in sg (11 and 4 percent of control at 10 and 20 days respectively). The corresponding values for wv were 41 and 12 percent and for rl 34 and 19 percent. It should perhaps be noted that although in all cerebellar mutants, binding with respect to control decreases over the period 10-20 days, the absolute values for (^{3}H) muscimol binding in these mutants show a significant increase (i.e. wv, $t = 3.69$, $p<0.01$; sg, $t = 3.13$, $p<0.02$; all 2 tailed Student's t-test). The concentration of (^{3}H) muscimol binding sites in preparations of cerebellar glomeruli was significantly higher (approximately 3-fold) than that of the homogenate from which they were prepared ($t = 3.36$, $p<0.05$, Student's t-test, Table 4).

(^{3}H) DHA binding. The Scatchard plot of (^{3}H) DHA binding (Figure 15) indicated that this ligand was bound to a single population of receptors with high affinity ($Kd = 0.26 + 0.5$ nM, $B_{max} = 86 \pm 7$ pmol per g protein at 20 days). Statistical analysis of the data in Figure 15 also indicated that the Kds for binding at 10 and 20 days were not significantly different (Figure 15 legend). (^{3}H) DHA binding developed mainly after birth (Figure 16). Significant binding was found in the cerebellum at 5 days. The maximum increase in binding occurred between 10 and 15 days and maximum levels of binding were achieved at 20 days (0.0888 pmol per cerebellum). Thereafter no significant changes in binding occurred. (^{3}H) DHA binding per cerebellum was significantly reduced in all mutants at 10 and 20 days except for jp (Figure 17, see also Table 5 for a summary of the analysis of variance of these data). Binding per cerebellum was most reduced in sg (20 and 14 percent control

at 10 and 20 days respectively). The corresponding values for wv were 46 and 22 percent and for rl, 48 and 19 percent. Although the cerebellar mutants show a reduction in binding relative to control between 10 and 20 days, the absolute binding figures show absolute increases (some of which are significant over this period (i.e. wv, $t = 2.87$, $p<0.05$; rl, $t = 1.7$, $p<0.1$ sg, $t = 6.46$, $p<0.01$; 2 tailed Student's t-test, using a method for non-homogenous variance (Winer, 1971) where appropriate).

(^{3}H) QNB binding. Examination of the kinetics of (^{3}H) QNB binding by Scatchard analysis (Figure 18) indicated that (^{3}H) QNB was bound to a single population of receptors with high affinity (Kd = 0.15 $\pm$ 0.01nM B_{max} = 162 $\pm$ 5 pmoles per g protein). In addition Figure 18 showed that the Kds at 10 and 20 days were not significantly different (Figure 18 legend). Most of the development of binding in the cerebellum occurred postnatally (Figure 19). Significant levels of binding were recorded at 5 days (0.099 pmol per cerebellum) and the maximum increase in binding occurred between 5 and 15 days. The development of (^{3}H) QNB binding was essentially complete by 20 days (0.773 pmol per cerebellum). (^{3}H) QNB binding was reduced in all the cerebellar mutants (Figure 20, see also Table 6 for a summary of the analysis of variance for this data). Binding was most affected in wv (40 and 36 percent of control at 10 and 20 days respectively) and sg (41 and 22 percent of control at 10 and 20 days respectively). Rl was the least affected of the cerebellar mutants (62 and 50 percent of control at 10 and 20 days). Jp mutants showed normal binding levels at 10 days and a 20 percent reduction in binding at 20 days. It should perhaps be pointed out that in all cerebellar mutants there was an increase in the concentration of binding sites compared to their controls (Figure 20). Examination of the kinetics of (^{3}H) QNB binding to mutant tissue (Table 7) indicated that the affinity constants for this binding were not significantly different from their individual controls.

In addition the Kd for binding to cerebral cortex (0.16 nM) was not significantly different from that for cerebellum although maximal binding was much higher in the cerebral cortex (B_{max} cerebral cortex = 1520 $\pm$ 100 pmol per g protein compared with B_{max} cerebellum = 162 pmol per g protein). Although in all cerebellar mutants there were no major changes in the amount of (^{3}H) QNB binding per cerebellum relative to controls at 10 and 20 days, there were significant increases in the absolute values of binding in wv, rl and sg cerebellum over this period (i.e. wv, $t = 7.65$, $p<0.01$; rl, $t = 4.03$ $p<0.01$; sg, $t = 10.14$ $p<0.01$). Table 4 shows that the concentration of (^{3}H) QNB binding sites in cerebellar glomeruli was not significantly different from that of the original homogenate from which these particles were prepared.

The data for the development of binding sites in normal mice and the comparison of binding sites in normal and mutant mice has been presented both in terms of binding per g protein (i.e. receptor concentration) and binding per cerebellum. However since the cerebellum is undergoing rapid growth over the period studied and because the cerebellar sizes of the mutants are much smaller than their controls, these two parameters provide differing information about the change in cerebellar receptor populations. In the development of binding sites in normal animals for example, the binding per cerebellum data show how the receptors change in number in this brain region. However the binding per g protein data, indicates how the receptor population changes during development in relation to the growth of the cerebellum. (i.e. if the increase in receptor population exceeds the change in cerebellar weight then one sees an increase in the receptor concentration). In the absence of data concerning the growth of the cerebellum such information could prove misleading. For example in the development of QNB binding there was no dramatic change in the concentration of these receptors in the cerebellum between 5 and 20 days, but the number of receptors in this brain region

increased 8-fold. When examining receptor populations in mutant animals the same problems arise. For example an increase in the concentration of binding sites (as seen for QNB binding) might be misleading if one did not know that mutant cerebella were much smaller than their controls. However such data provides information concerning the possible location of binding sites. If the concentration of binding sites is increased in mutant cerebella, this may indicate that those cells remaining have a higher proportion of receptors than those cells lost. The binding per cerebellum data for the mutants indicates whether there has been a net loss of receptors from the brain region as a result of the mutation. Thus if the receptors were unaffected by the mutations the binding per cerebellum data would be identical to controls whereas the concentration of binding sites would be markedly increased. To avoid confusion, the discussion will be limited to binding per cerebellum unless indicated otherwise.

FIGURE 12 Scatchard plot of (^{3}H) muscimol binding to the total particulate fraction of 10 day (circles) and 20 day (triangles) mouse cerebellum. Each point is the mean of 2 experiments (range < 15 percent). The binding parameters, calculated from these data by linear regression (Edwards, 1967) were as follows:

10 days, Kd = 16.4 $\pm$ 3.4 nM, B_{max} = 310 $\pm$ 48 pmol per g protein;

20 days Kd = 13.0 $\pm$ 1.4 nM, B_{max} = 1282 $\pm$ 105 pmol per g protein.

The regression coefficients for the linear regressions were 0.86 and 0.95 for 10 and 20 days respectively. The difference between the Kds was not statistically significant ($t = 0.51$, $p<0.50$, 2 tailed Student's t-test).

FIGURE 12

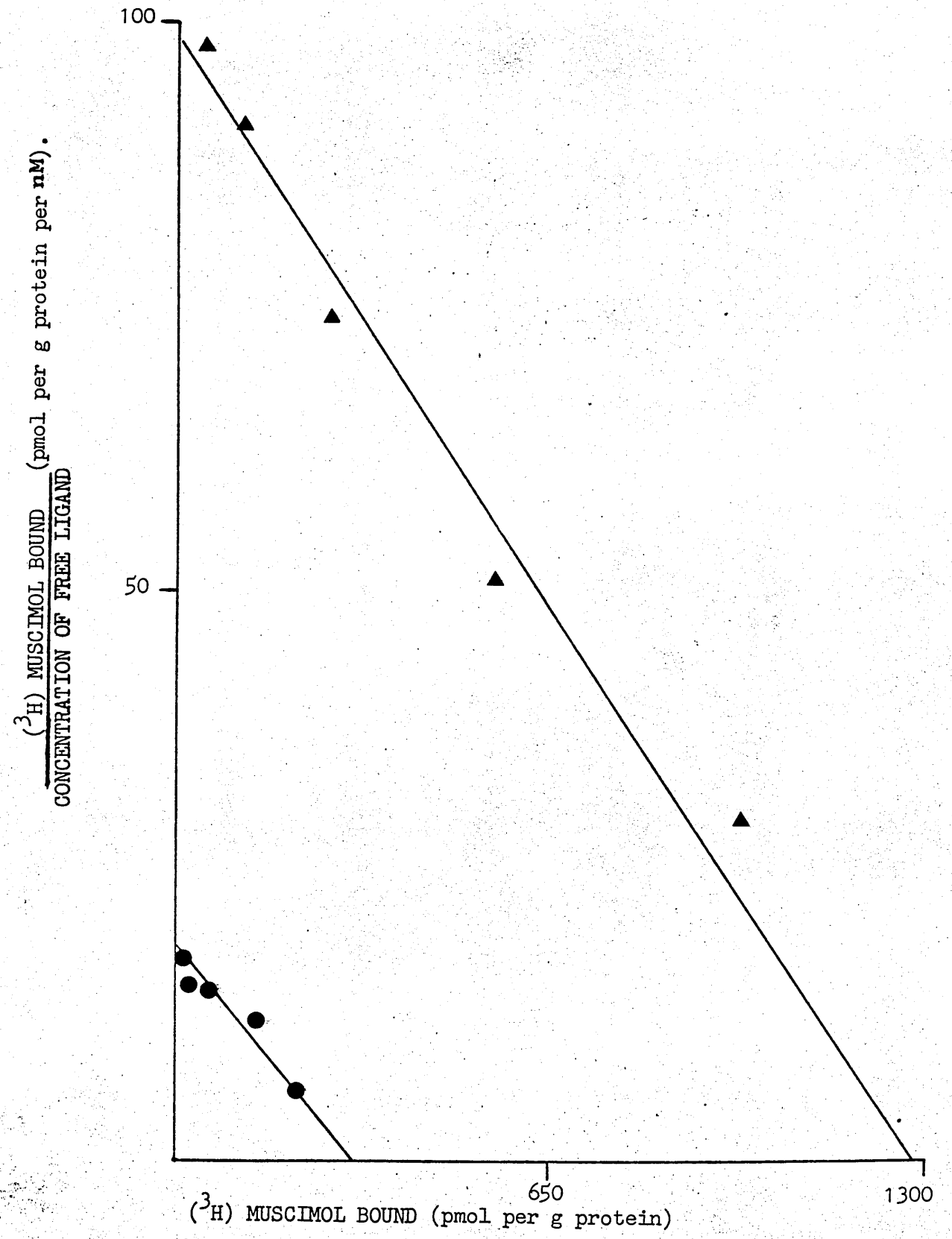

100
50
650
1300
(^{3}H) MUSCIMOL BOUND / CONCENTRATION OF FREE LIGAND (pmol per g protein per nM).
(^{3}H) MUSCIMOL BOUND (pmol per g protein)

FIGURE 12a Time course of (^{3}H)muscimol binding. Cerebellar tissue from 20 day animals was incubated with (^{3}H)muscimol for 1,3 and 10 minutes before centrifugation. The results are from 2 experiments and are expressed as means ± range.

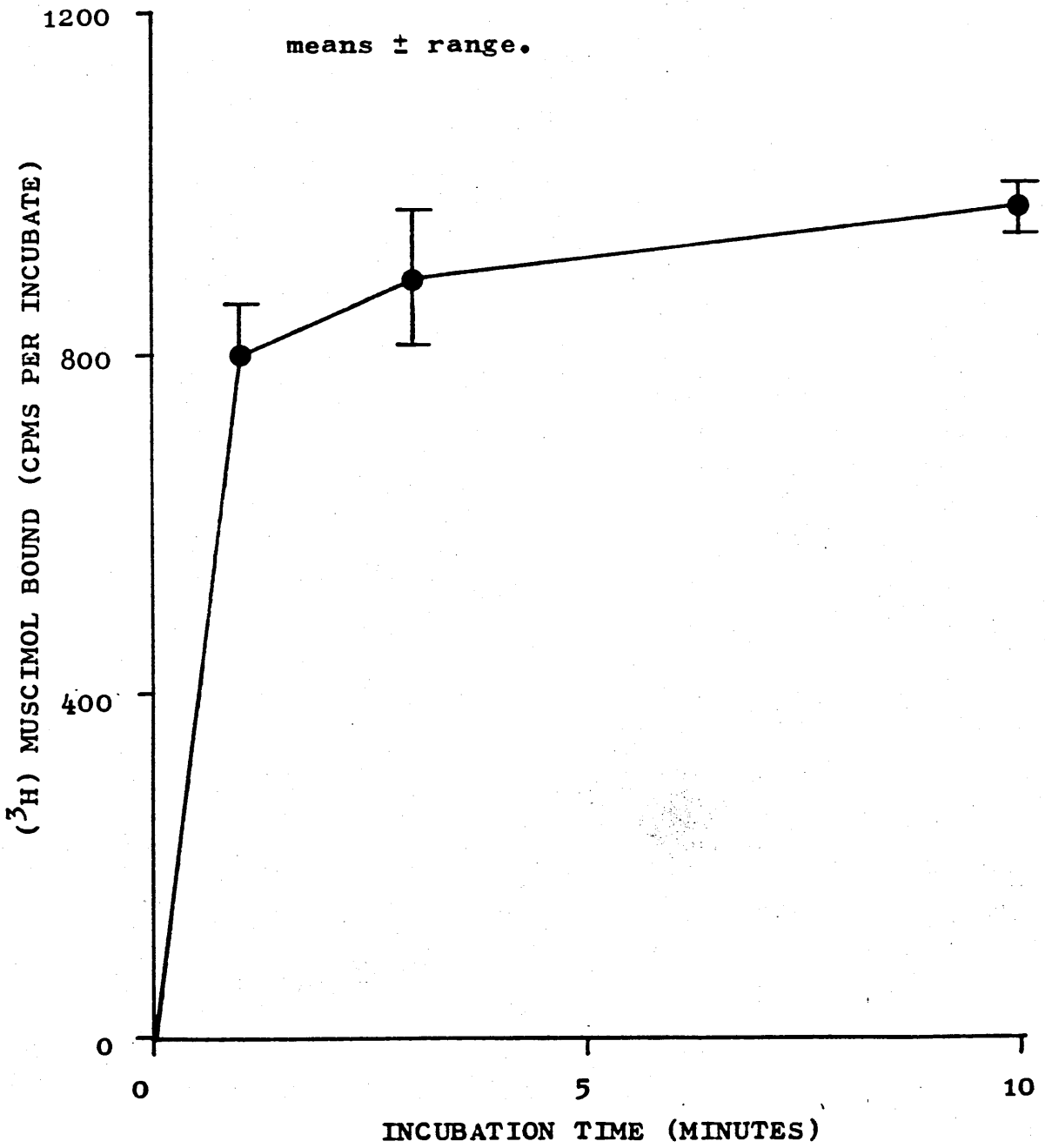


FIGURE 13 The development of (^{3}H) muscimol binding in normal mouse cerebellum. Results plotted as means ± S.E.M. n = 5. Unbroken line shows data expressed as pmol per cerebellum; broken line shows data as pmol per g protein.

FIGURE 13

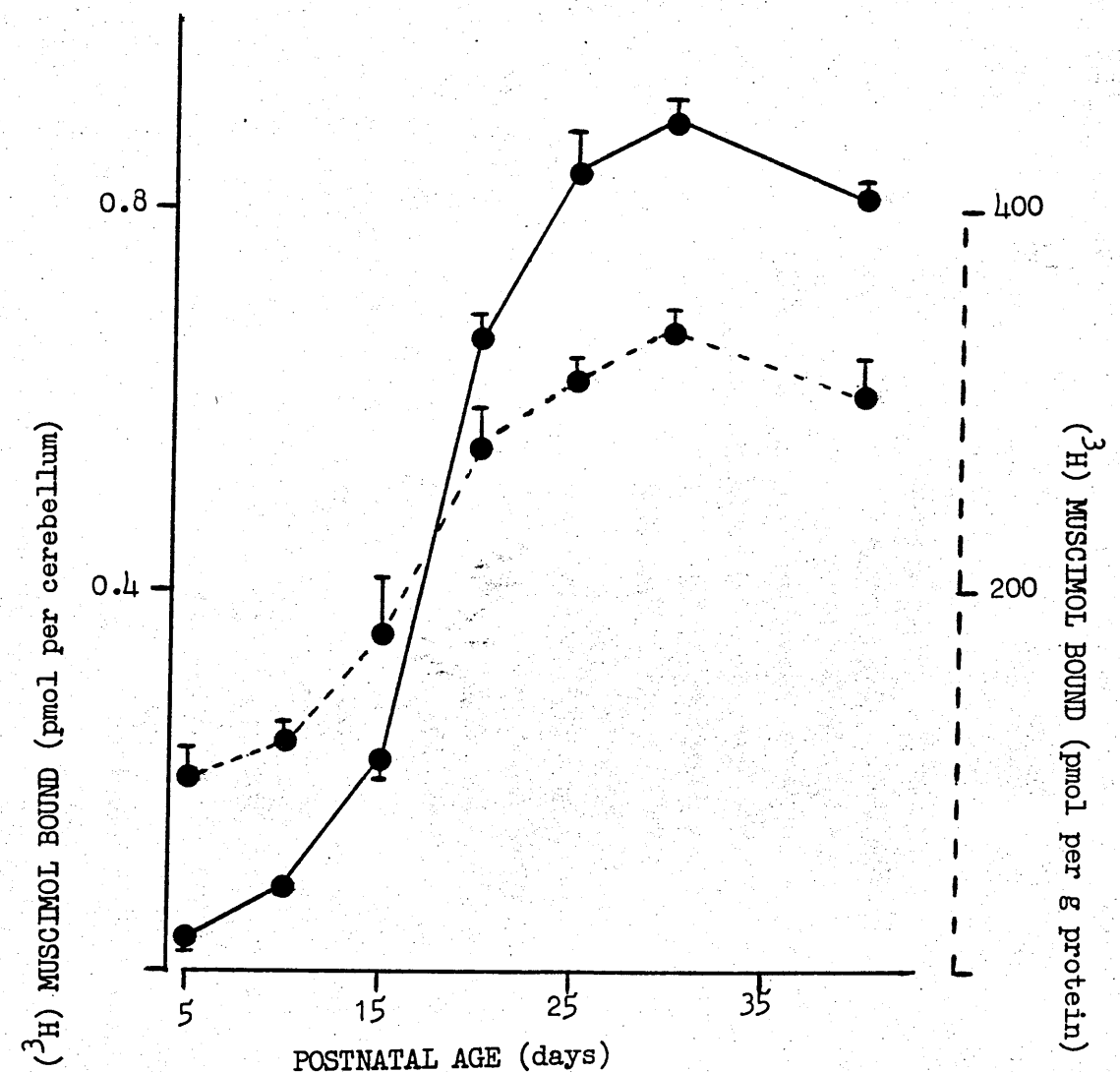

0.8
0.4
400
200
5
15
25
35
POSTNATAL AGE (days)
(^{3}H) MUSCIMOL BOUND (pmol per cerebellum)
(^{3}H) MUSCIMOL BOUND (pmol per g protein)

Figure 14 A summary of the data for (^{3}H) muscimol binding to "freeze thawed" total particulate fraction from the cerebella of weaver, reeler, staggerer and jimpy mutants at age 10 and 20 days (as indicated below bars). The data are expressed as mean percent control ± s.e.m. (n = 5), both as binding per cerebellum (open bars), and binding per unit protein (shaded bars). The appropriate controls were taken from the following sources: wv, rl and jp controls were normal B6/CBA stock; sg controls were non-carriers from sg stock, identified by their dilute coat marker (see page 113 for further details).

The absolute control values at age 10 days were as follows: wv, rl and jp controls, 0.080 ± 0.012 pmol per cerebellum, 99 ± 10 pmol per g protein; sg controls, 0.070 ± 0.011 pmol per cerebellum, 67 ± 7 pmol per g protein. At 20 days the corresponding values were: wv, rl and jp controls, 0.533 ± 0.025 pmol per cerebellum, 273 ± 14 pmol per g protein; sg controls 0.344 ± 0.021 pmol per cerebellum, 152 ± 11 pmol per g protein.

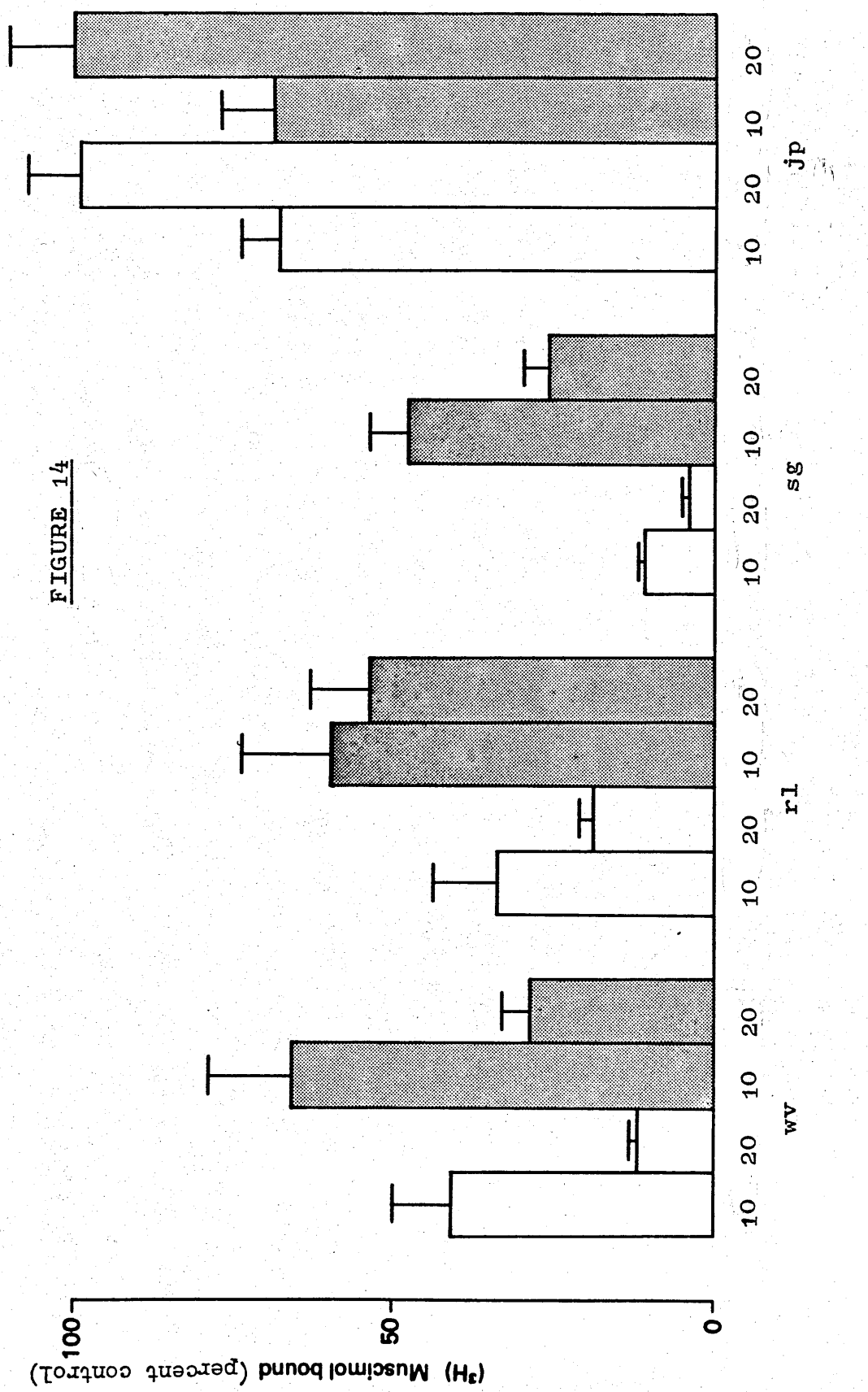

(³H) Muscimol bound (percent control)
100
50
0
10 20 10 20
wv
10 20 10 20
rl
10 20 10 20
sg
10 20 10 20
jp

FIGURE 14

FIGURE 15 Scatchard plot of (^{3}H) DHA binding to the total particulate fraction of 10 day (circles) and 20 day (triangles) mouse cerebellum. Each data point represents the mean of 2 determinations from duplicate experiments (range 15 per cent). The binding parameters, calculated from this data by linear regression (Edwards, 1967), were as follows: 10 days, Kd = 0.19 ± 0.03 nM, B_{max} = 66 ± 4 pmol per g protein; 20 days, Kd = 0.26 ± 0.05 nM, B_{max} = 86 ± 7 pmol per g protein; The regression coefficients for the linear regressions were 0.90 and 0.88 for 10 and 20 days, respectively.

The difference between the Kds was not statistically significant ($t = 1.14, p > 0.25$, 2 tailed Student's t-test).

FIGURE 15

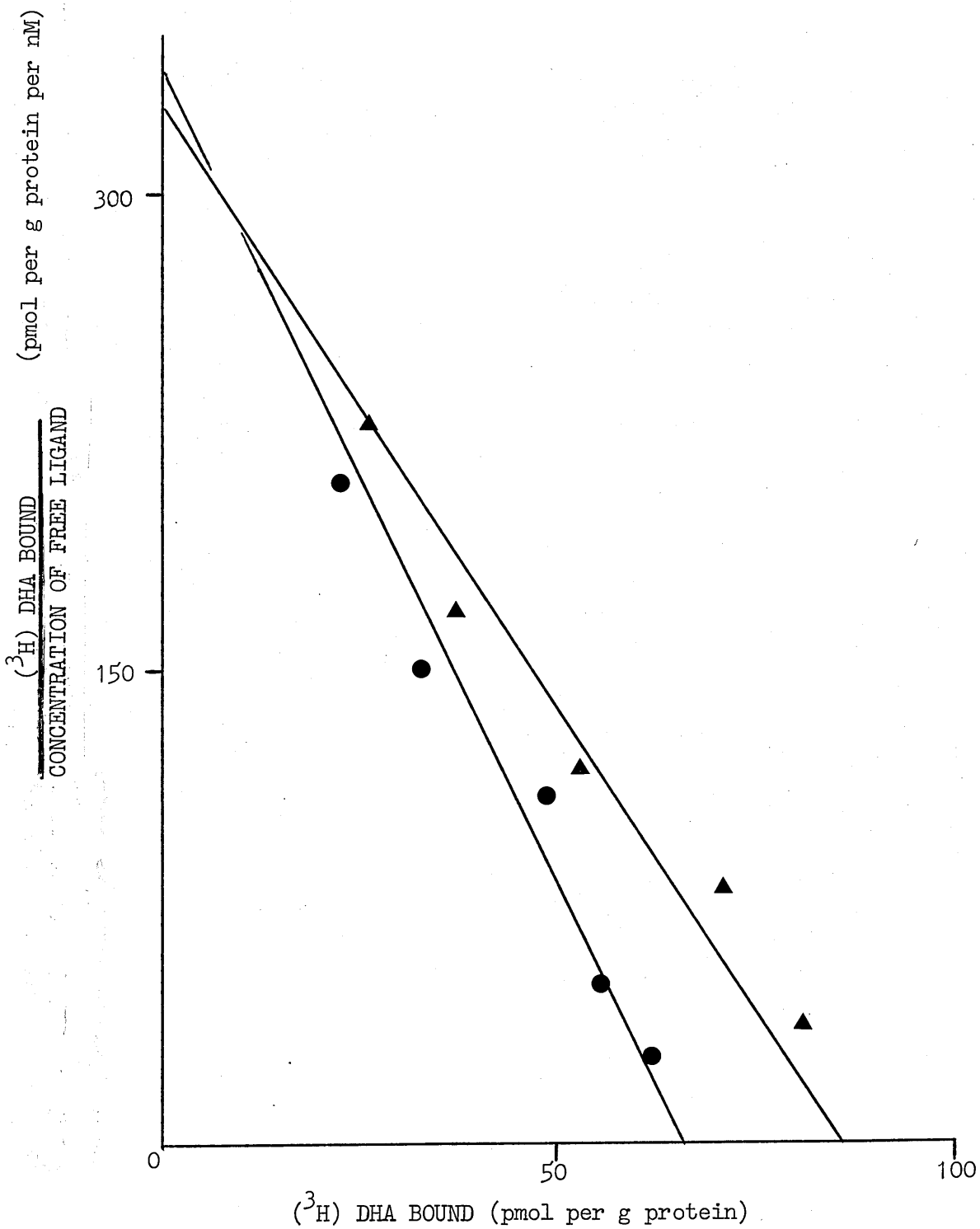

(pmol per g protein per nM)
(^{3}H) DHA BOUND / CONCENTRATION OF FREE LIGAND
300
150
0
50
100
(^{3}H) DHA BOUND (pmol per g protein)

FIGURE 15a Time course of (^{3}H)DHA binding. Cerebellar tissue from 20 day animals was incubated with (^{3}H)DHA for 1,5,12 and 25minutes before filtering. The results are from 2 experiments and are expressed as means ± range.

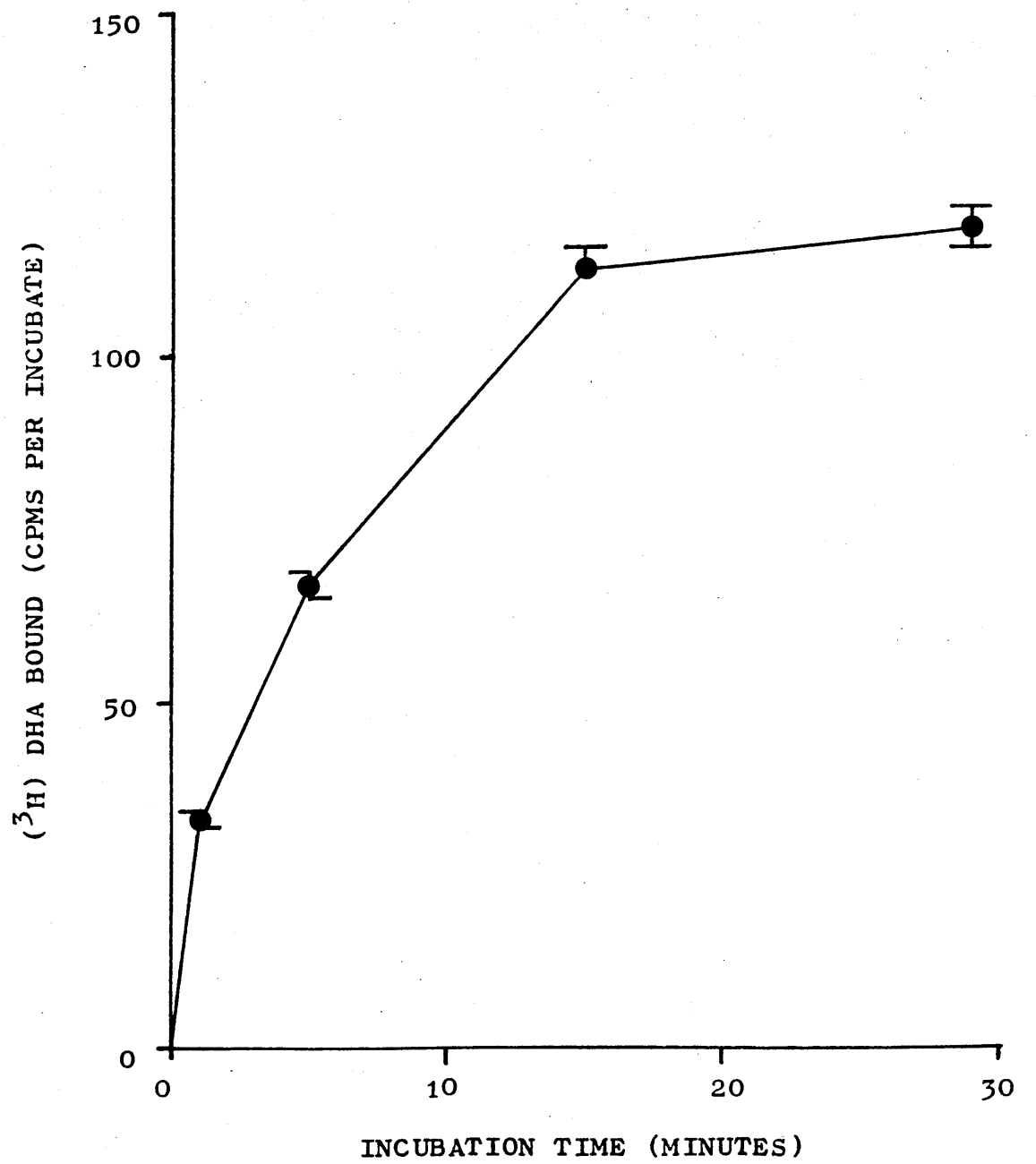


FIGURE 16 The development of (^{3}H) DHA binding in normal mouse cerebellum. Results are plotted as means ± S.E.M. n = 6 unless indicated otherwise in parantheses. Unbroken line shows data expressed as pmol per cerebellum; broken line shows data as pmol per g protein.

FIGURE 16

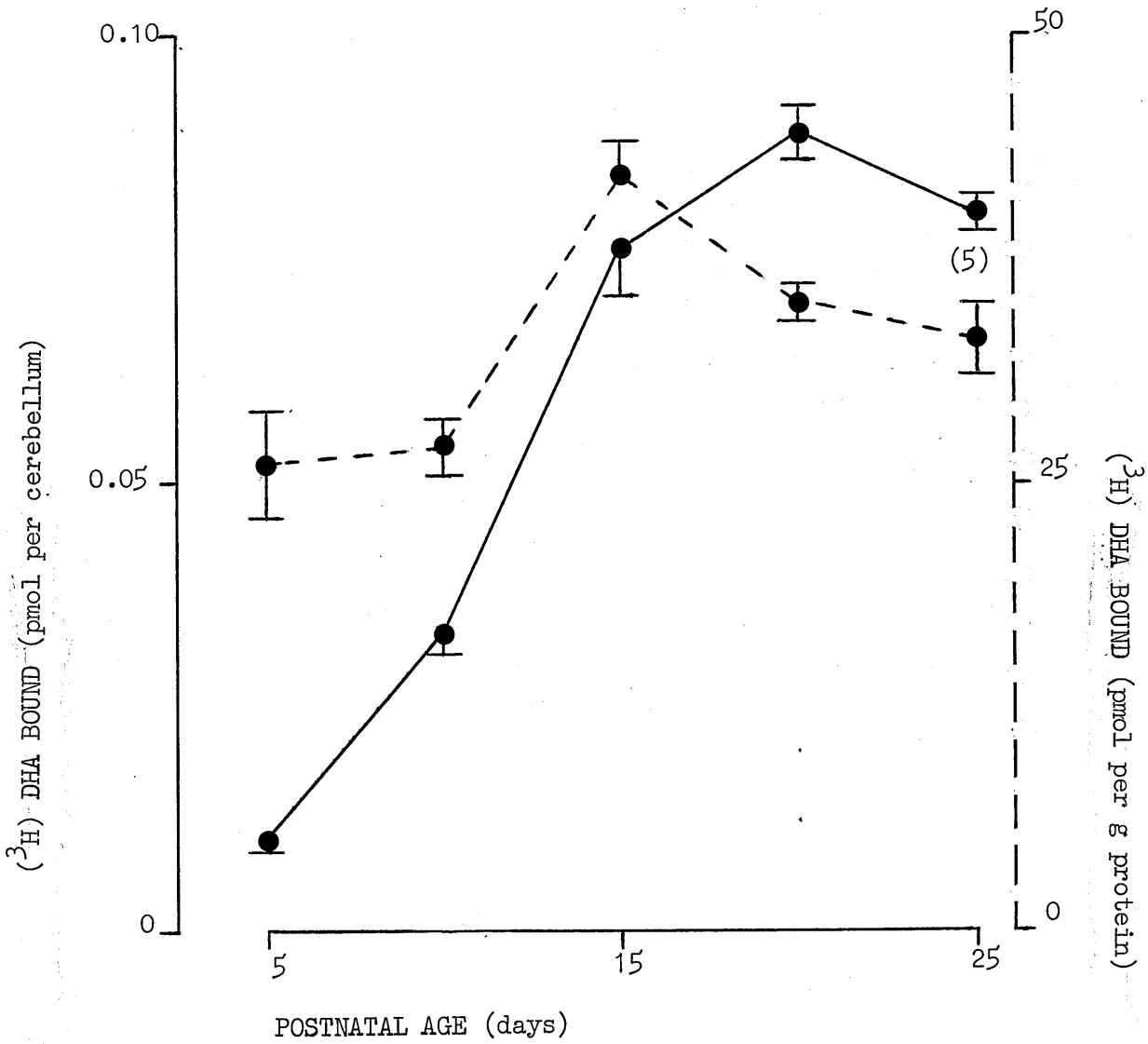

0.10
0.05
0
(^{3}H) DHA BOUND (pmol per cerebellum)
50
25
0
(^{3}H) DHA BOUND (pmol per g protein)
(5)
5
15
25
POSTNATAL AGE (days)

Figure 17 A summary of the data for (^{3}H) DHA binding to the total particulate fraction from the cerebella of weaver, reeler, staggerer and jimpy mutant mice at age 10 and 20 days (as indicated below bars). The data are expressed as mean percent control ± s.e.m. (n = 5), both as binding per cerebellum (open bars), and binding per unit protein (shaded bars). The absolute control values at age 10 days were as follows: wv controls, 0.0285 ± 0.0011 pmol per cerebellum, 30.7 ± 1.0 pmol per g protein; rl controls, 0.0250 ± 0.0033 pmol per cerebellum, 24.5 ± 3.9 pmol per g protein; sg controls, 0.0268 ± 0.0021 pmol per cerebellum, 39.6 ± 2.6 pmol per g protein; jp controls, 0.0274 ± 0.0039 pmol per cerebellum, 31.6 ± 3.9 pmol per g protein. At 20 days the values were: wv controls, 0.0888 ± 0.0043 pmol per cerebellum, 42.1 ± 1.5 pmol per g protein; rl controls, 0.0888 ± 0.0043 pmol per cerebellum, 42.1 ± 1.5 pmol per g protein, sg controls, 0.0665 ± 0.0031 pmol per cerebellum, 43.3 ± 1.5 pmol per g protein, jp controls, 0.0874 ± 0.0041 pmol per cerebellum, 43.7 ± pmol per g protein.

FIGURE 17

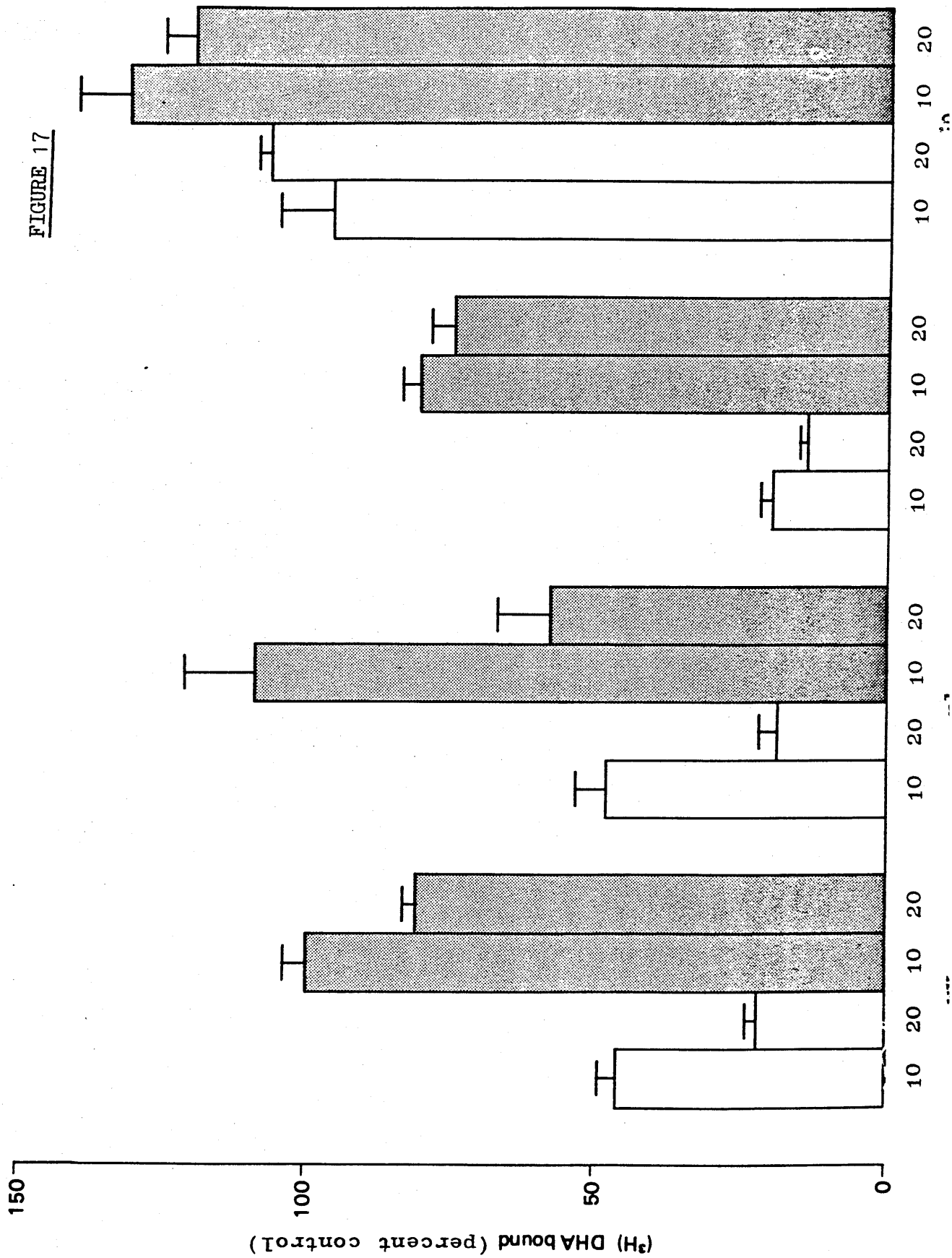

150
100
50
0
(^{3}H) DHA bound (percent control)
10
20
10
20
10
20
10
20
10
20
10
20
10
20
10
20

FIGURE 18 Scatchard plot of (^{3}H) QNB binding to cerebellar homogenates from 10 day (triangles) and 20 day (circles) mouse. Each point is the mean of 4 experiments (S.E.Ms < 10 percent). The binding parameters calculated from this data by linear regression (Edwards, 1967) were as follows: 10 days, Kd = 0.14 ± 0.01 nM, B_{max} = 131 ± 4 pmol per g protein; at 20 days Kd = 0.14 ± 0.03 nM, B_{max} = 118 ± 11 pmol per g protein. The regression coefficients for the linear regressions were 0.97 and 0.99 at 10 and 20 days, respectively. The difference between the Kds was not statistically significant ($t = 0.037$ $p < 0.25$, 2 tailed Student's t-test).

FIGURE 18

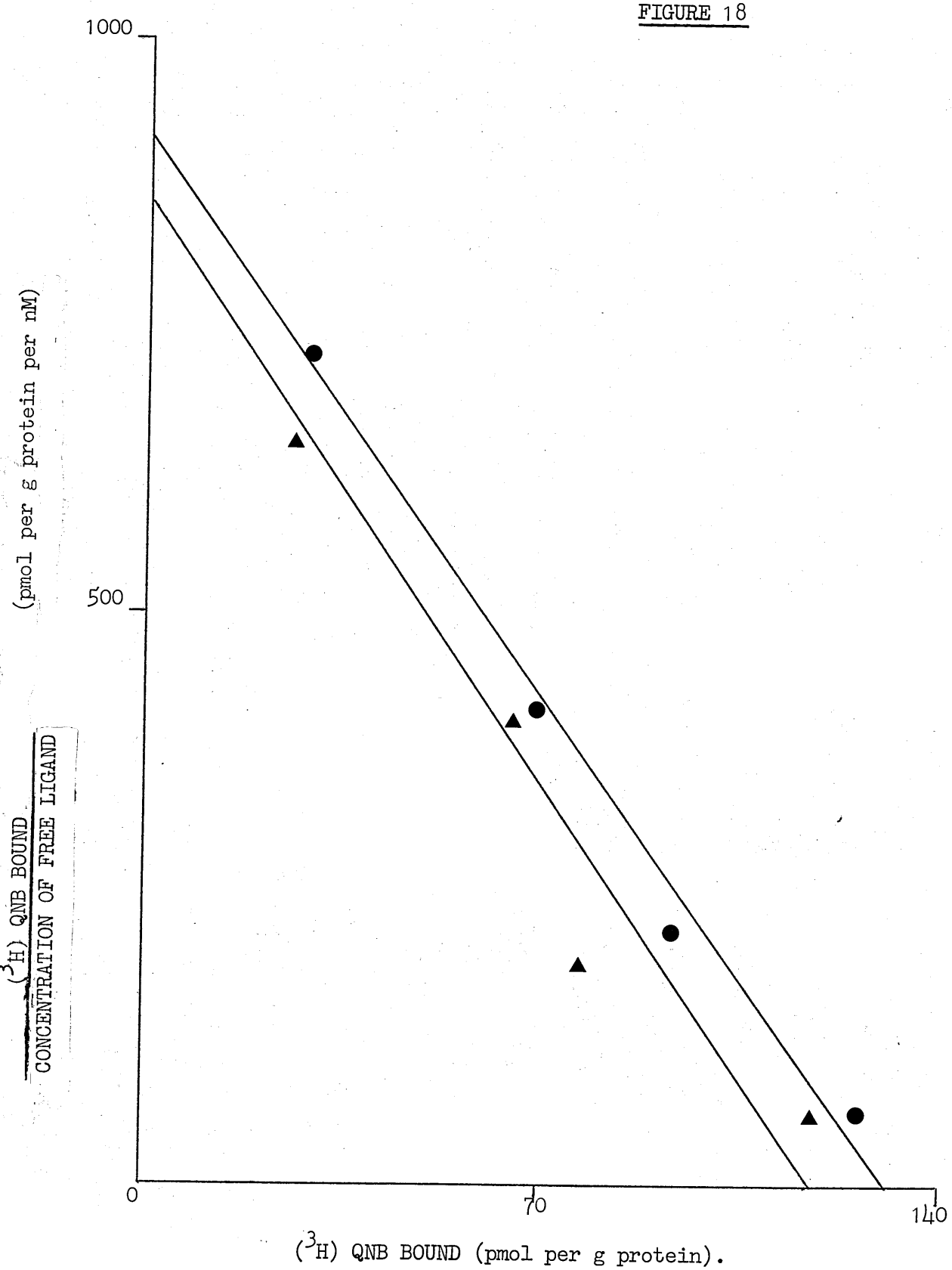

1000
500
0
70
140
(pmol per g protein per nM)
(^{3}H) QNB BOUND / CONCENTRATION OF FREE LIGAND
(^{3}H) QNB BOUND (pmol per g protein).

FIGURE 18a Time course of (^{3}H)QNB binding. Cerebellar homogenates from 10 day mice were incubated with (^{3}H)QNB for 5,10,20 and 60 minutes before filtering. The results are from 2 experiments and are expressed as means ± range.

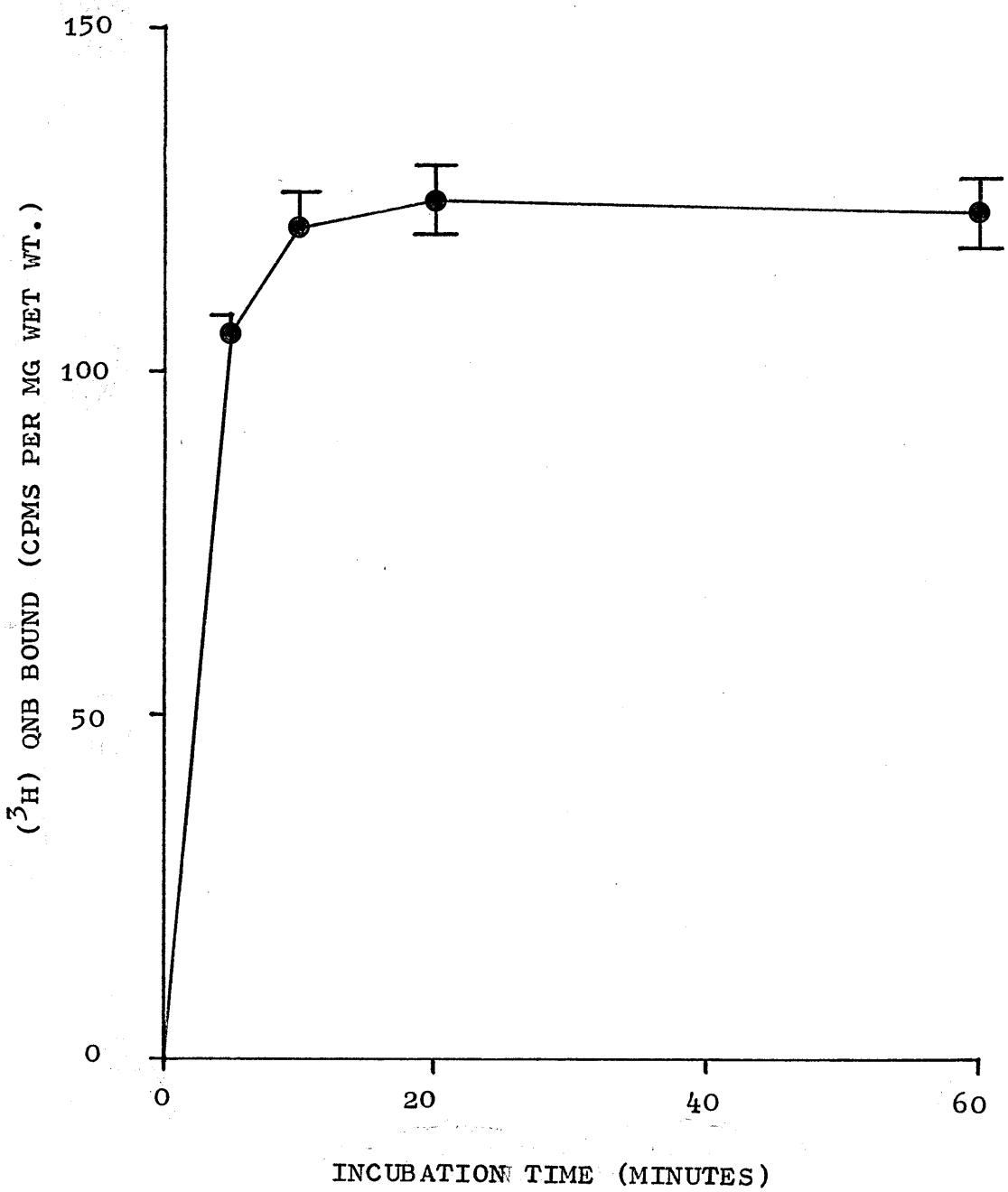


FIGURE 19 The development of (^{3}H) QNB binding in normal mouse cerebellum. Results plotted as means ± S.E.M., n = 6 unless otherwise indicated in parentheses. Unbroken line shows data expressed as pmoles per cerebellum; broken lines shows data as pmoles per g protein. The cerebellar wet wts. of these animals are as follows : 5 days, 12 ± 0.5 mg; 10 days, 32 ± 1.3 mg; 15 days, 41 ± 1.4 mg; 20 days, 50 ± 1.1 mg; 25 days, 51 ± 1.8 mg.

FIGURE 19

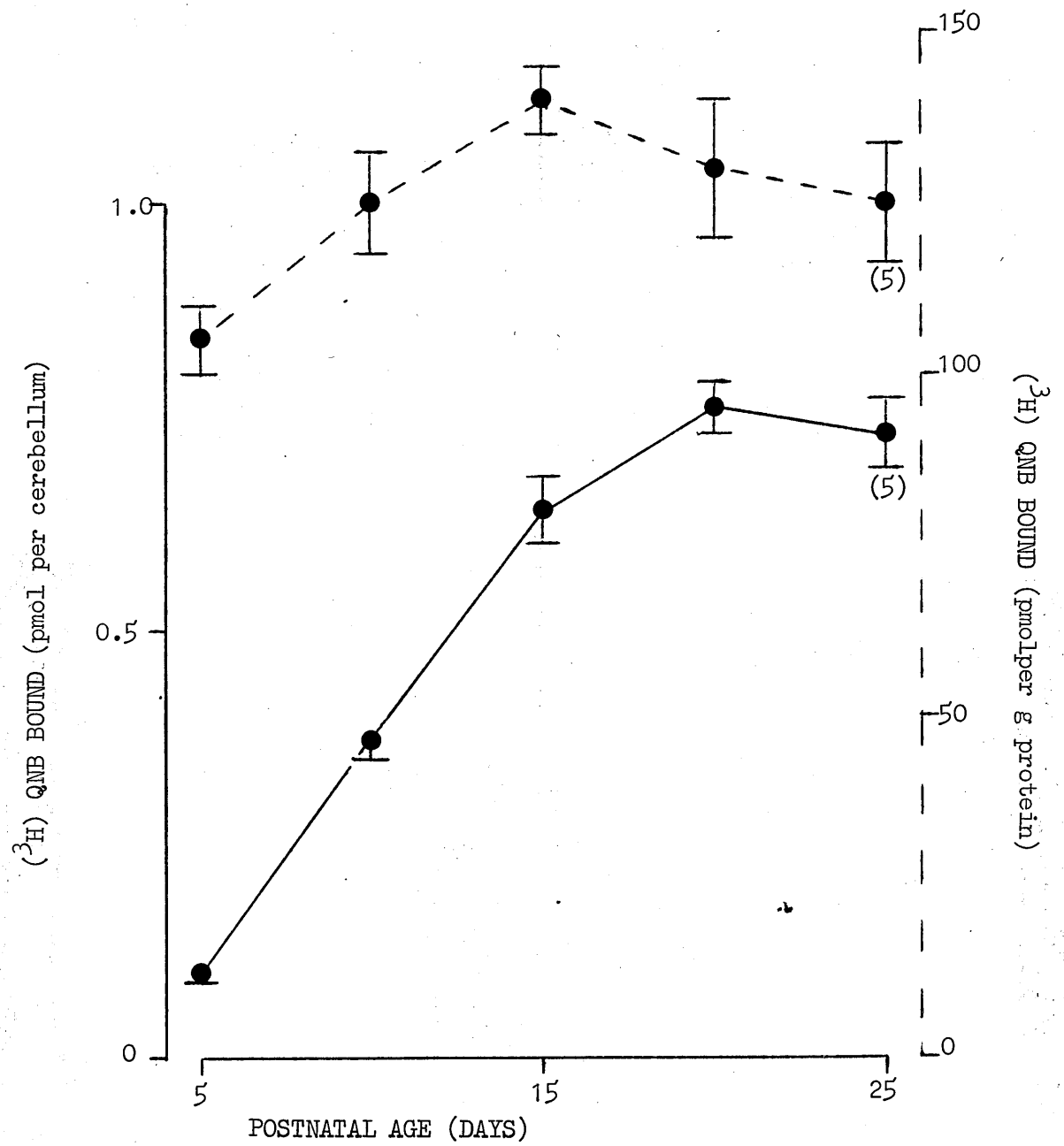

150
1.0
(5)
100
(5)
0.5
50
0
0
5
15
25
POSTNATAL AGE (DAYS)
(^{3}H) QNB BOUND (pmol per cerebellum)
(^{3}H) QNB BOUND (pmolper g protein)

Figure 20 A summary of the data for (^{3}H) QNB binding to cerebella homogenates of weaver, reeler, staggerer and jimpy mutants at age 10 and 20 days (as indicated below bars). The data are expressed as mean percent control ± s.e.m. (n = 6) in the form (^{3}H) QNB bound per cerebellum (open bars) and (^{3}H) QNB bound per unit protein (shaded bars). The absolute control values at age 10 days were as follows: for wv and jp, 124 ± 7 pmoles per g protein, 0.373 ± 0.023 pmol per cerebellum; rl control, 118 ± pmol per g protein; 0.373 ± 0.011 pmol per cerebellum; sg control (grey), 134 ± 7, pmol per g protein, 0.384 ± 0.011 pmol per cerebellum. The mean cerebellar wet weights (mg) for the mutants were: wv, 15 ± 0.8, sg 9 ± 0.2; rl, 14 ± 0.6; jp, 31 ± 1.8. At 20 days the control values were wv and jp control, 130 ± 9 pmoles per g protein, 0.773 ± 0.022 pmoles per cerebellum, rl control, 118 ± 5 pmoles per g protein, 0.659 ± 0.016 pmoles per cerebellum; sg control, 156 ± 11 pmoles per g protein, 0.649 ± 0.032 pmoles per cerebellum. The mean cerebellar wet weights (mg) for the mutants were: wv, 16 ± 0.4; sg, 8 ± 0.2; rl, 16 ± 0.5; jp, 40 ± 0.8.

FIGURE 20

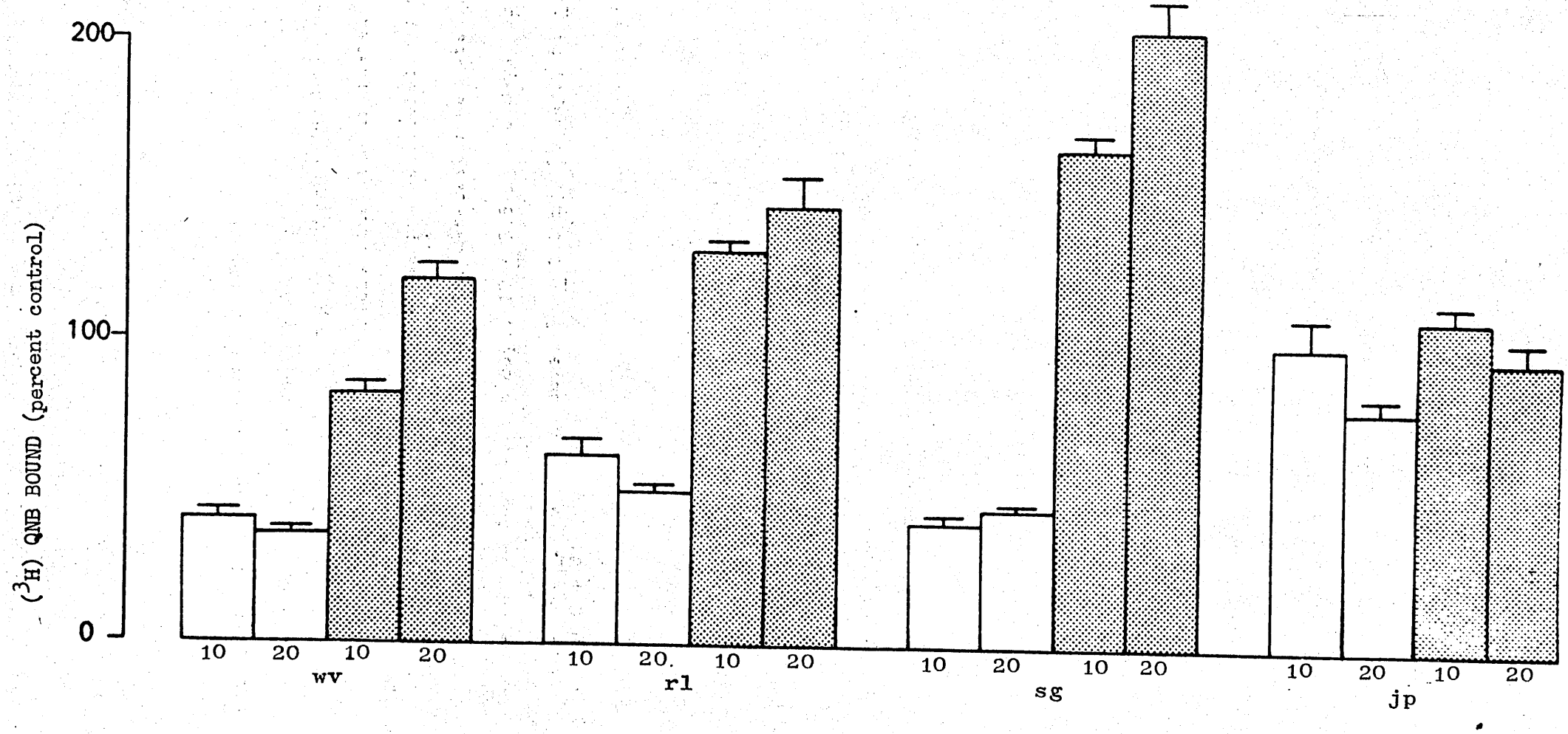

200
100
0
(^{3}H) QNB BOUND (percent control)
10
20
10
20
wv
10
20
10
20
rl
10
20
10
20
sg
10
20
10
20
jp

FIGURE 21 A summary of the binding data from figures 13,16,19,plus the cerebellar weight data from figure 19(legend), is given below. The data are expressed as a percentage of the value from 25 day animals, to allow a comparison of the developmental profiles of (^{3}H)QNB, (^{3}H)DHA and (^{3}H)muscimol binding and cerebellar growth.

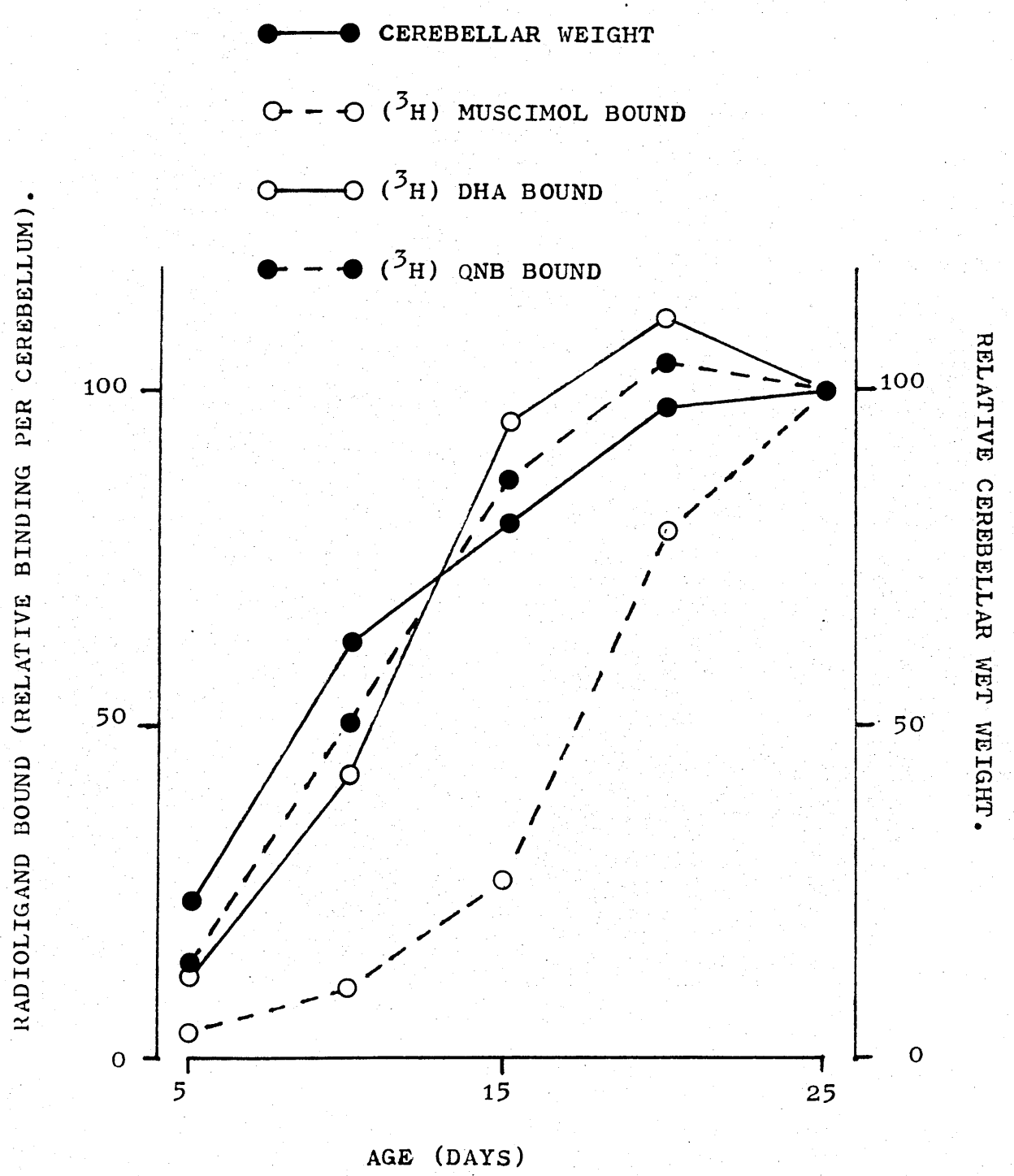


TABLE 3 Summary of analysis of variance performed on (^{3}H) muscimol binding (per cerebellum) in mutants and their controls.

	AGE (10 vs 20 days)	MUTANT vs CONTROL
wv	28.2	33.7
sg	17.7	48.5
rl	32.5	24.4
jp	381	0.03[a]

The experiments were designed for 2 x 2 factorial analysis of variance. However a significant difference in cell variances was observed in all comparisons ($p<0.05$, from variance ratio distribution statistic). (Homogeneity of variance is a prerequisite for analysis of variance (Edwards, 1967)). Thus the simple effects of age and mutation were computed using a modification of analysis of variance, which takes into account the heterogeneity of variance, outlined in Winer (1971).

The F ratios are given above for the simple effects of age and mutation ($n = 5$, 1 and 19 degrees of freedom). All F ratios are highly significant ($p<0.001$) unless indicated otherwise.

[a] $p>0.25$ (not significant).

TABLE 4 The binding of (^{3}H) muscimol and (^{3}H) QNB to fractions enriched in cerebellar glomeruli, values expressed as mean $\pm$ S.E.M. (n = 3).

	AMOUNT OF LIGAND BOUND (pmol per g protein)	
LIGAND	TOTAL HOMOGENATE	GLOMERULI FRACTION
(^{3}H) MUSCIMOL	431 $\pm$ 159	1076 $\pm$ 295*
(^{3}H) QNB	152 $\pm$ 30	142 $\pm$ 10

*Significantly different from total homogenate value $t = 3.362$ $p < 0.05$ (2-tailed Student's t-test).

TABLE 5 Summary of analysis of variance performed on (^{3}H) DHA binding (per cerebellum) in mutants and their controls.

	AGE (10 vs 20 days)	MUTANT vs CONTROL
wv	18.7	38.5
sg	9.5[b]	66.4
rl	19.9	38.0
jp	437	0.06[a]

The experiments were designed as outlined in Table 3 legend. However a significant difference in cell variances was observed ($p<0.05$ from variance ratio distribution statistic). Thus F ratios for the simple effects of mutation and age were computed (see Table 3 legend) (n = 5, 1 and 19 degrees of freedom). All F ratios given above are highly significant ($p<0.001$) unless indicated otherwise.

[a] $p>0.25$ (not significant) [b] $p<0.01$.

TABLE 6 Summary of analysis of variance performed on (^{3}H) QNB binding (per cerebellum) in mutants and their controls.

	AGE (10 vs 20 days)	MUTANT vs CONTROL	INTERACTION
wv*	14.8	45.3	-
sg*	10.7[b]	47.5	-
rl	170	255	41
jp	147	8.4[a]	9.5[a]

The F ratios are given for a 2 x 2 factorial design (n = 6, 1 and 20 degrees of freedom) unless indicated otherwise.

Significant F ratios for interaction means that the combined effects of mutation and age were non additive.

*Where no interaction values are shown, a significant difference in cell variances was observed ($p<0.05$, from variance ratio distribution statistic) (see Table 3 legend). In such cases the F ratios for the simple effects of mutation and age were computed (see Table 3 legend for details) (n = 6, 1 and 23 degrees of freedom). All F ratios given above are highly significant ($p<0.001$) unless otherwise indicated.

[a]$p<0.025$ [b]$p<0.005$.

TABLE 7 A summary of binding parameters for (^{3}H) QNB binding to normal and mutant mouse cerebellar material. The Kd and Bmax values were obtained from Scatchard plots of data (n = 8) from duplicate experiments. The line of best fit for the Scatchard plot was determined by linear regression (Edwards, 1967).

Mouse strain (Age in days)	Apparent Kd (nM±SEE)	Apparent Bmax (pmoles/gprotein ± SEE)	Correlation cooefficient
wv/wv (20)	0.13 ± 0.01	163 ± 4	.99
B6/CBA (20)	0.11 ± 0.02	130 ± 9	.93
sg/sg (15)	0.08 ± 0.02	291 ± 27	.85
C57BL/6 (grey) (15)	0.09 ± 0.02	138 ± 11	.89
rl/rl (12)	0.11 ± 0.01	159 ± 5	.98
C57BL/10 (12)	0.13 ± 0.02	155 ± 13	.92
B6/CBA cerebellum (20)	0.14 ± 0.01	162 ± 5	.99
B6/CBA cerebral cortex (20)	0.16 ± 0.02	1520 ± 100	.95

TABLE 8 Body weights of normal and mutant mice used in the QNB binding study (Figure 20) at age 10 and 20 days. Values expressed as mean ± S.E.M. (n = 5 or 6).

	BODY WEIGHT (g)	
ANIMAL TYPE	10 days	20 days
B6/CBA	5.9 ± 0.2	9.1 ± 0.2
C57BL/6 (dilute)	5.9 ± 0.3	7.7 ± 0.4
wv	4.7 ± 0.3	6.1 ± 0.4
sg	4.5 ± 0.3	5.8 ± 0.3
rl	5.1 ± 0.5	6.0 ± 0.4
jp	4.5 ± 0.6	5.2 ± 0.2

TABLE 9 A summary of the binding per cerebellum data shown in figures 14, 17 and 20. Values are expressed as mean ± S.E.M. (see individual figures for further details).

MUTATION (age in days)	RADIOLIGAND BINDING PER CEREBELLUM (PER CENT CONTROL)		
	(^{3}H) MUSCIMOL	(^{3}H) DHA	(^{3}H) QNB
wv (10)	41 ± 9	46 ± 3	40 ± 3
wv (20)	12 ± 1	22 ± 2	36 ± 2
rl (10)	34 ± 10	48 ± 5	62 ± 4
rl (20)	19 ± 2	19 ± 3	50 ± 2
sg (10)	11 ± 1	20 ± 1	41 ± 3
sg (20)	4 ± 1	14 ± 1	44 ± 1
jp (10)	68 ± 6	96 ± 9	100 ± 10
jp (20)	99 ± 8	107 ± 2	80 ± 3

3.4 Discussion

Muscimol binding. The maximal amount of muscimol binding in 20 day mouse cerebellum in this study (1.3 pmol per mg protein, Figure 12) is similar to that observed by Snodgrass (1978), B_{max} 3.3 pmol per mg protein and Beaumont et al. (1978), B_{max} (high affinity binding) 0.7 pmol per mg protein in the rat cerebral cortex and by Wang et al. (1979), B_{max} (high affinity binding) 2.1 pmol per mg protein, for mouse cerebral cortex. Although none of these studies examined binding in the cerebellum, Beaumont et al. (1978) found that binding in the cerebellum was twice as high as in cerebral cortex. Assuming an identical Kd in both brain regions the B_{max} in cerebellum would approximate to 1.4 pmol per mg protein. It should be noted that the studies of Snodgrass (1978), Beaumont et al. (1978) and Wang et al. (1979) were carried out on crude synaptic membrane preparations, whereas this study was performed on the total particulate fraction. One would expect the concentration of receptors in the preparation used here to be lower than that of crude synaptic preparations. The level of specific binding reported here for mouse cerebellar total particulate fraction, up to 80 percent, compares with over 80 percent reported by Beaumont et al. (1978) for rat brain membranes. Snodgrass (1978) reported a figure of 65 percent for specific binding to rat brain membranes and the higher level of non specific binding may be the result of less thorough washing. Snodgrass (1978) only washed the preparation once after the incubation and centrifugation, whereas in this study and that of Beaumont et al. (1978) the pellet was rinsed twice. The Kd for muscimol binding to mouse cerebellar tissue at 20 days (13 $\pm$ 1 nM, Figure 12) was similar to that reported by Wang et al. (1979) (9 nM) for high affinity binding to mouse cerebral cortex. However this figure is higher than that reported by Snodgrass (1978) (2.7 nM) and intermediate between high and low affinity binding sites (Kd = 2.2 $_{n}M$ and 60 $_{n}M$) found by Beaumont et al. (1978) for rat cerebral cortex. The biphasic nature of binding reported

by Beaumont et al. (1978) and Wang et al. (1979) was not apparent in these studies (Figure 12) indicating that over the range of concentrations used muscimol was bound to a single population of high affinity binding sites. Since there was no significant difference in the affinity of binding at 10 and 20 days it is probable that the changes in binding occurring during development were due to a change in the number of receptors and not due to a change in the affinity of the receptor for the ligand. Because of the large amounts of material required to examine the kinetics of binding, information concerning the Kd of muscimol binding to mutant material was not available. However since the binding of muscimol to normal cerebellum did not indicate the presence of more than one high affinity site for this ligand it has been assumed in this discussion that changes in muscimol binding to the mutants were due to changes in the number of receptors.

The developmental profile of muscimol binding (Figure 13) closely resembles that reported for sodium independent GABA binding to rat cerebellar material (Coyle & Enna, 1976) which adds further weight to the hypothesis that muscimol binds to GABA receptors (see section 1.7). The majority of GABA receptors in the cerebellum are thought to be localised on the granule cell dendrites (postsynaptic to the Golgi cell axons) in the cerebellar glomeruli, because the granule neurons are known to be receptive to GABA (see section 1.4) and are by far the most numerous neuronal type in the cerebellum (Eccles et al. 1967). Additional evidence for the localisation of the majority of GABA receptors on the granule cells comes from studies of virally induced granule cell deficient hamsters (Simantov et al. 1976) and mutant mice (sg, wv and rl) (Olsen & Mikoshiba, 1978) which show a severe reduction in sodium independent GABA binding. In the study of Olsen & Mikoshiba

it is also reported that GABA receptor binding in the cerebellum of the nervous mutant (nr) which has a deficit of Purkinje neurons (Sidman & Green, 1970) was not significantly different from control animals. Thus these authors concluded that GABA receptors on Purkinje cells make up only a small fraction of the GABA receptors in the cerebellum. Further evidence for this idea comes from the study of Roffler-Tarlov (1979). This study examined (^{3}H) GABA binding to the cerebellum of the nervous mutant over the period of Purkinje cell death, which starts around day 15, and showed that there was no marked loss in binding sites over this period. The reduction in binding seen in agranular cerebella (Simantov et al.1976; Olsen & Mikoshiba, 1978) suggests that a large proportion of the GABA receptors are associated with granule neurons. This is supported by the finding that (^{3}H) muscimol binding is significantly enriched in cerebellar glomeruli fractions (Table 4) and the report by Olsen & Mikoshiba (1978) which showed that purified granule cell fractions contained large numbers of (^{3}H) GABA receptors. Thus it seems probable that the development of muscimol binding is mainly a reflection of the maturation of GABAergic synapses on the granule cells. The finding that the major increase in muscimol binding is between 15 and 20 days supports this idea, since in the mouse the cerebellar glomeruli approach maturity around day 15 although the maturation of the glomeruli is not complete until around day 35 (Larramendi, 1969). A number of studies have suggested that receptor formation precedes synaptogenesis (Woodward et al. 1971; Crain & Bornstein, 1974), and the finding that significant levels of muscimol binding (0.028 pmol per cerebellum) are found in 5 day mouse cerebellum supports this idea since the earliest GABAergic synapses appear around day 7 (Larramendi 1969).

The reduction in muscimol binding to the cerebellar material from wv, rl and sg is similar to that observed by Olsen & Mikoshiba (1978) for sodium independent GABA binding. This is further confirmation of the validity of the use of muscimol to examine GABA receptors.

Interpretation of the mutant data reported here poses some problems. Although the most marked feature of the cerebellum of these animals is the loss of granule neurons, other anomalies occur, both as a consequence of granule cell loss and in certain instances, possibly as a more direct effect of the mutation (Rakic & Sidman, 1973 a,b; Sotelo, 1975 a,b; Sidman, 1968; Rakic, 1976; see also section 1.3). Despite this problem, the finding that muscimol binding is severely reduced in the cerebella of the animals (Figure 14 and Table 3) suggests that a large proportion of the muscimol binding deficit is due to the loss of binding sites on granule neurons. Of the cerebellar mutants, rl is least affected (19 percent control at 20 days) and this might be predicted, since these animals have significant numbers of normal granule cells (Sidman, 1968).

Binding is similarly affected by the wv mutation (12 percent control at 20 days). The slightly lower figure for the wv mutant may be the result of the larger granule cell deficit seen in wv compared with rl (Sidman, 1968). The sg mutant shows the largest binding deficit (4 percent control at 20 days). Whether the increased reduction in sg compared with wv is the result of Purkinje cell loss in these animals is unclear. However, the finding that the levels of the GABA synthesising enzyme (glutamate decarboxylase) are more reduced in sg (20 percent control at 21 days) than wv (30 percent control) (Beart & Lee, 1977) indicates that GABAergic neuron activity is markedly more affected in sg compared with wv, possibly as a result

of Purkinje cell loss. The finding that muscimol binding is not significantly affected by the jp mutation (Figure 14) is consistent with the normal neuronal organisation of the jp cerebellum (Hirano et al. 1969; Sidman et al. 1964).

In all cerebellar mutants the deficit in muscimol binding increased in severity over the period 10-20 days (Figure 14), which is perhaps an indication that the processes responsible for the reduction in binding continue to operate over this interval. This process may be the continuing granule cell death/short fall in available granule neurons, which occurs maximally between 10 and 21 days (see Section 1.3). Both Olsen & Mikoshiba (1978) and Simantov et al. (1976) attempted to estimate the percentage of total GABA receptors on the granule cells using the results of sodium independent GABA binding to agranular cerebellar tissue. This has not been attempted here since it is not possible to calculate what effects the reduction in granule cells has on the GABA receptor populations of the remaining cell types.

DHA binding. The level of DHA binding observed in this study (B_{max} 86 $\pm$ 7 pmol per g protein at 20 days, Figure 15) compares with the values of 110 pmol per g protein (for crude membrane fraction) and 5.1 pmol per g wet wt. (for total particulate fraction), reported for DHA binding to rat cerebellum (Alexander et al. 1975; Johnson & Hallman, 1978). The Kds reported for this binding by Alexander et al. (1975) and Johnson & Hallman (1978) (6.7 nM and 1.6 nM, respectively) were higher than the value reported here (0.26 $\pm$ 0.05 nM, Figure 15). However Bylund (1978)

using similar concentrations of radioligand and isoproterenol as a displacer (see Section 1.7 for the importance of displacer choice in β-adrenoceptor binding studies), obtained a Kd of 0.5 nM for DHA binding to guinea pig cerebral cortex. That the Kds for DHA binding at 10 and 20 days did not differ significantly (Figure 15 legend) suggests that the changes in binding seen during development were due to a change in the number of receptors and not due to a change in the affinity of the receptor for the ligand. Because of the large amounts of material required to examine the kinetics of binding, information concerning the affinity of DHA binding to mutant cerebellar material was not available. However since the binding of DHA to developing normal cerebellum did not indicate the presence of more than one high affinity site for this ligand, it has been assumed here that changes in mutant DHA binding were due to changes in the number of binding sites.

Significant amounts of DHA binding were found in 5 day animals (0.010 ± 0.001 pmol per cerebellum)(Figure 16), and by analogy with the rat, it seems likely that noradrenergic synapse formation occurs prior to this age (Yamamoto et al. 1977). There is no direct evidence concerning the neurotransmitter function of these early binding sites in the mouse cerebellum. However, Harden et al. (1977) examined the ontogeny of β-adrenoceptors in the rat cerebral cortex using the ligand (^{125}I) iodohydroxybenzylpindolol ((^{125}I) IHYP). They found that the development of these binding sites was coincident with that of catecholamine stimulated adenylcyclase activity. In addition, Woodward et al. (1971) showed that rat Purkinje neurons were responsive to iontophoretically applied noradrenalin as early as birth. Thus it is probable that these early receptors in the mouse cerebellum are capable of functioning.

The sharp increase in DHA binding between 10 and 15 days coincides with the translocation and maturation of the climbing fibres (Larramendi, 1969). Although the noradrenergic fibres are not considered as climbing fibres (Bloom et al. 1971), they are similar in a number of respects. For example, both types of fibre synapse with Purkinje neurons on the dendritic thorns (Palay & Chan-Palay, 1974) and fluorescence studies indicate that as with climbing fibres, noradrenergic fibres are initially located around the cell body during development, but subsequently come to occupy the primary and secondary Purkinje cell dendrites in the molecular layer (Yamamoto et al. 1977; Bloom et al. 1971). Thus it may be that the noradrenergic fibres may undergo translocation similar to that documented for the climbing fibres (Larramendi, 1969). The time course for the development of DHA binding seen in this study is also paralleled by an increase in tyrosine hydroxylase activity (the enzyme responsible for the control of catecholamine synthesis) with a six-fold increase occurring in the mouse cerebellum between 10 and 21 days (Beart & Lee, 1977). This gives further support to the idea that the development of DHA binding is closely associated with the development of noradrenergic functioning in the cerebellum.

DHA binding to total particulate fractions from the cerebella of wv, rl and sg mutants was markedly reduced (Figure 17), but was not significantly different from control value for material from jp mutants. The latter finding is consistent with the normal neuronal organisation in the cerebellum of jp mutants (Sidman et al. 1964; Hirano et al. 1969). If one assumes that the Purkinje cells are responsible for most of the DHA binding in the cerebellum (see

Section 1.4) then it is not surprising that DHA binding was reduced in sg (14 percent control at 20 days, Figure 17) since these animals have a severe Purkinje cell deficit (Herrup & Mullen, 1979), and those Purkinje neurons which survive appear immature and stunted (Sidman, 1968; Landis & Sidman, 1978; see also Section 1.3). That adrenergic functioning is impaired in sg, is supported by the finding that tyrosine hydroxylase levels are much reduced (approximately 50 percent control at 20 days in the cerebella of these animals) (Beart & Lee, 1977). In addition using histofluorescence techniques the noradrenergic fibres of sg appear as disorganised matted tangles (Landis et al. 1975). The reduction in binding in wv and rl mutants is more difficult to explain since these animals have relatively normal numbers of Purkinje neurons (Sidman, 1968), although the dendritic arbor of these neurons is affected by the mutations (see Section 1.3). Reduced levels of tyrosine hydroxylase in wv mutant cerebellum has been reported (Beart & Lee, 1977) although the normal pattern of noradrenergic innervation of Purkinje neurons appears to remain (Landis et al. 1975). It seems likely therefore that in wv cerebellum the deficit in DHA binding is due to reduced noradrenergic functioning. Presumably this is brought about indirectly by the effects of granule cell loss on the Purkinje cells, since the wv mutation is not thought to act directly in the Purkinje neurons (see Section 1.3). Since the final result of the wv and rl mutations on cerebellar organisation is very similar (Sidman, 1968) one might suggest that the reduction in DHA binding to the cerebellum is also the result of a deficit in noradrenergic functioning.

Unfortunately, there are no data for tyrosine hydroxylase activity

in the rl mutant. However Landis et al. (1975) examined the noradrenaline levels in wv, rl and sg. The levels in sg and wv, consistent with tyrosine hydroxylase activity, were markedly reduced (approximately 50 percent control) while in rl the noradrenaline levels were not significantly different from control values. The reason for the difference is noradrenaline levels in wv and rl are not readily apparent, since the final effect of these mutations on the Purkinje cells is very similar (Sidman, 1968). It should perhaps be noted that there appears to be a major anomaly in the data presented by Landis et al. (1975). The cerebellar weights for their mutants, sg, wv and rl (27, 39 and 30 mg wet wt.) contrast markedly with those used here (8, 16 and 16 mg wet wt), and with those reported by other workers including Mallet et al. (1976); Beart & Lee (1977) and Mariani et al. (1977). These differences were not the result of the mutations being carried on different backgrounds since identical strains were used in this study.

Another mechanism by which a reduction in DHA binding might be achieved is by noradrenergic hyperinnervation. Hyperinnervation of the rat cerebellum (produced by the neurotoxic agent 6-hydroxydopamine) has been shown to reduce DHA binding in this region (Johnson & Hallman, 1978; Harden et al.1979). However this hyperinnervation, which increased noradrenaline levels two-fold, only reduced (^{3}H) DHA binding to 80 percent of control. Since as already outlined there is no evidence to suggest that the cerebella of wv, sg or rl are hyperinnervate, it seems unlikely that such a phenomenon could account for the reduction in DHA binding seen in this study. Thus despite the findings of Landis et al. (1975), that

noradrenaline levels in rl cerebellum were normal, it seems probable that the reduction in DHA binding is the result of reduced noradrenergic functioning in this mutant.

In all cerebellar mutants the deficit in DHA binding relative to controls, increased in severity over the period (10-20 days, Figure 17), which is perhaps an indication that the processes responsible for the reduction in binding continue to operate over this interval. This increasing deficit is most pronounced in wv (where binding fell from 46 percent control at 10 days to 22 percent control at 20 days) and in rl (where the corresponding fall in binding was from 48 to 19 percent control). This is consistent with the idea that the effect of the granule cell deficit (which occurs mainly during this period) on Purkinje cell development may be a major factor in reducing the number of DHA binding sites in wv and rl cerebellum. In the sg mutant the fall in binding relative to control between 10 and 20 days was much smaller (falling from 20 percent at 10 days to 14 percent at 20 days; Figure 17). This might be taken as an indication that the granule cell loss plays a smaller part in the DHA binding deficit seen in sg. This is in accord with the suggestion that Purkinje cell loss might be the main cause of reduction in DHA binding in sg cerebellum.

There is little evidence for β-adrenoceptors on neurons other than the Purkinje cells in the cerebellum (Tebecis, 1974). Although a number of studies have shown that glial cells possess β-adrenergic ligand binding sites and catecholamine stimulated adenyl cyclase (Clark & Perkins, 1971; Gilman & Nirenberg, 1971; Maguire et al. 1976; Harden et al. 1976). However 6-hydroxydopamine which specifically destroys noradrenergic nerve terminals significantly

affects β-adrenergic ligand binding (Johnson & Hallman 1978; Harden et al. 1979) and catecholamine sensitive adenylate cyclase (Kalisher et al. 1973) which suggests that these receptors are in close association with noradrenergic nerve terminals, i.e. postsynaptic specialisations on neurons. The studies of Atlas et al. (1977) using the fluorescent β-adrenergic ligand 9-amino acridinepropranolol (9-AAP) failed to show any major localisation over cells other than neurons in the cerebellar and cerebral cortex. Further evidence for the mainly neuronal localisation of β-adrenoceptors comes from the sg mutant data. DHA binding in the cerebellum of these mutant animals is severely reduced (14 percent control at 20 days) although considerable astrocytic proliferation occurs (Hirano & Dembitzer, 1976; Lee et al. 1977) indicating that DHA binding to glia is probably not important in this study.

QNB binding. The amount of QNB binding in 20 day old mouse cerebellum seen in this study (130 ± 9 pmol per g protein, Figure 19) compares with values of 34 pmol per g protein (for crude membrane preparation) (Yamamura & Snyder, 1974a) and 51 pmol per g protein (total homogenate) (Kobayashi et al. 1978) for the rat cerebellum. Mallol et al. (1979) examined the binding of QNB to rat cerebellar homogenates using a variety of techniques and reported binding levels as high as 312 pmol per g protein using Millipore filtration and centrifugation techniques (see Section 1.7). They attributed the lower levels of binding reported by Yamamura & Snyder (1974a) and Kobayashi et al. (1978) partly to the use of glass fibre filters. However in this laboratory no significant difference was observed in QNB binding when the glass fibre filtration method was compared with the centrifugation method (East unpublished). The apparent

Kd for binding to mouse cerebellar tissue was slightly lower (0.15 ± 0.03 nM, Table 7) than that reported for the rat (0.4 nM) (Yamamura & Snyder, 1974). That the apparent Kd for QNB binding to cerebellar homogenates was not significantly affected by development (Figure 18) or as a result of the mutations (Table 7) indicates that the increases in QNB binding reported here were due to alterations in the numbers of receptors for the radioligand. In addition the finding that the Kds for cerebral cortex and cerebellum were not significantly different (Table 7) indicates that the QNB receptors are probably the same in both brain regions. This finding is in agreement with work recently published by Yavin & Harel (1979) for the rabbit although these authors found a much higher Kd (1.5 nM).

The developmental profile (maximal increase occurring between 5 and 15 days) coincides with a major period of synaptogenesis in the mouse cerebellum (Larramendi, 1969). However the time course of development of QNB binding in the cerebellum appears to have important interspecies variation . Yavin & Harel (1979) found that in rabbit the development of QNB binding was complete by postnatal day 10, which corresponds roughly to day 5 or 6 in the mouse (based on cerebellar weights relative to adult values). At this age the cerebellum is very immature (around 25 percent of adult wet wt.) and synaptogenesis is just beginning (Larramendi, 1969). Thus the total development of QNB binding precedes synaptogenesis in the rabbit cerebellum and because of this Yavin & Harel (1979) suggested that these receptors might be implicated in neurogenesis.

The development of QNB binding in the brain of chick and rat is closely followed by the development of acetylcholinesterase (AchE) and cholineacetyltransferase (CAT) (Enna, Yamamura & Snyder, 1976; Coyle & Yamamura, 1976).

Thus the data suggest that in the whole brain QNB binding may be associated with cholinergic synaptogenesis. Beart & Lee (1977) have shown that over the period 10-20 days the total CAT activity of the mouse cerebellum increases two-fold. In the present study QNB binding increased by a similar amount over the same time period (Figure 19). The evidence suggests that as for whole brain the development of QNB binding in the cerebellum is accompanied by the ontogenesis of a cholinergic marker indicating that QNB may be the result of synaptogenesis which contrasts with what occurs in the rabbit cerebellum. Although the data are not comprehensive, similar events seem to occur during the development of the human cerebellum (Brooksbank et al. 1978).

Although the development of QNB binding parallels that of the cholinergic marker CAT, muscarinic binding sites are located mainly in the molecular layer, where there are few cholinergic fibres (Rotter et al. 1979b).

The function and precise cellular location of these receptors is unknown. However Rotter et al. (1979b) listed four structures in the adult rat molecular layer on which these muscarinic receptors might be located. These were the non receptive parts of the Golgi and granule cells (parallel fibres) in the molecular layer (see Section 1.4 for evidence of their cholinoceptive nature). Rotter et al. (1979b) also considered that the Purkinje cells might also account for this binding since cholinoceptive Purkinje neurons have been reported (Crawford et al.1966). They also suggested that muscarinic receptors might occur on the climbing fibres since the pattern of development of

these fibres closely corresponds to that seen for (^{3}H) PBCM binding, i.e. starting in the deep medullary layer and moving through the granular layer to terminate in the molecular layer after 14 days (Rotter et al. 1979c). In addition Rotter et al. (1979c) postulated that this binding in the molecular layer might not be functional and might be a histogenetic expression of some primitive cholinergic mechanism.

That QNB binding is reduced by 50-60 percent of control in the agranular mutants, wv, rl and sg, suggests that a significant number of QNB binding sites are not localised on granule neurons, or their parallel fibres. This is further substantiated by the significant increases in the concentration of QNB receptors seen in the cerebella of all these mutants at 20 days (Figure 20). Also the finding that QNB binding sites are not enriched in cerebellar glomeruli fractions (Table 4) supports the idea that a large proportion of these sites are on cells other than granule neurons. The possibility that large numbers of QNB binding sites are located on the Purkinje neurons is difficult to reconcile with the mutant data. The QNB binding levels in wv and sg are similar (Figure 20) despite the fact that sg has a severe Purkinje cell deficit (Herrup & Mullen, 1979). Thus of the four structures which might account for muscarinic binding sites as proposed by Rotter et al. (1979b) the Golgi neurons and the climbing fibres seem the more likely candidates. However if the major site of QNB binding were the climbing fibres, one might expect the increase in QNB binding to level out considerably after 15 days, since the study of Larramendi (1969)

indicates that these fibres attain their adult location and connections before this age. Thus of the alternatives, the location of muscarinic binding sites on Golgi neurons seems the most tenable.

QNB binding in the jp mutant, which has an apparently normal cerebellum in terms of synaptic organisation (Sidman et al. 1964; Hirano et al. 1969), was only slightly reduced (80 percent control at 20 days, Figure 20). This slight reduction in binding may have been the result of a nutritionally based retardation of development since these animals have lower body and cerebellar wts. (Figure 20 legend and Table 8).

It has been suggested that axonal growth is affected by the paucity of myelin in these animals (Webster et al. 1976, see also Section 1.3) and therefore the deficit in QNB binding might be the result of impaired neuronal input.

In all agranular mutants there is no large reduction in the amount of QNB binding relative to controls over the period 10-21 days. This may indicate that the initial effects of these mutations, i.e. those occurring before 10 days, play a larger role in producing the binding deficit than the larger reduction in granule cells seen between 10 and 20 days (Sidman 1968; Miale & Sidman, 1961). Another possibility is that reorganisation of the cerebellum might account for the absence of a further binding deficit over this period. For example, mossy fibres form more than the usual number of contacts with Golgi cells in the wv mutant (Rakic & Sidman, 1973b). Such contacts might stimulate receptor formation.

Whatever the site or sites of QNB binding in the cerebellum, the neuromodulatory role of these receptors, if any, remains to be established. However it seems unlikely that they play a major role in neurotransmission at the mossy fibre-granule cell dendrite synapse, since autoradiographic evidence strongly suggests that these sites are nicotinic (Hunt & Schmidt, 1978).

A comparison of the binding of the three ligands. The high concentration of muscimol binding sites in mouse cerebellum 1280 pmol per g protein, (Figure 12) is in agreement with the wide range of evidence which indicates that all the major classes of neurons in the cerebellum receive GABAergic contacts (see Section 1.4). The level of binding of DHA is an order of magnitude smaller (86 pmol per g protein), but this is consistent with DHA binding sites being sited predominantly on the Purkinje neurons which account for less than 0.1 percent of cerebellar neurons (see Section 1.1). The similarly low level of QNB binding (130 pmol per g protein) coupled with the fact that these receptors do not appear to be localised adjacent to any cholinergic input (Rotter et al. 1979b) would seem to indicate that these receptors do not play a major role in cerebellar neurotransmission.

The developmental profiles of binding for the three ligands show some marked differences. Muscimol binding for example occurs latest in development (Figure 21) and the binding levels of

this ligand increase much faster than cerebellar growth. In contrast the increases seen in QNB and DHA binding during development closely parallel the increase in cerebellar wet weight. These differences are reflected in the finding that only the concentration of muscimol binding increased markedly during development (Figures 13, 16 and 19). The possible reasons for these differences have been outlined in the previous discussion but it is worth repeating these points. Both QNB and DHA binding sites appear to be localised on cells other than granule neurons. These cells (i.e. Purkinje and interneurons) mature relatively early in development and form a large number of synapses during the period of granule cell proliferation and migration (i.e. before 15 days) (Larramendi, 1969; Miale & Sidman, 1961). Since granule cell proliferation accounts for the increase in cerebellar size, this may explain why the developmental profiles for DHA and QNB binding closely follow the cerebellar growth curve (Figure 21). The majority of muscimol binding is thought to be on granule cell GABA receptors. The granule neurons continue to achieve their final locations up to around 15 days (after which cerebellar growth slows considerably (Figure 21, Miale & Sidman, 1961). However the synaptic development of the cells may continue up to 35 days (Larramendi, 1969). This may explain why muscimol receptor binding levels continue to increase after cerebellar growth has finished.

Although there is a large discrepancy in the binding levels of DHA and muscimol, the development of these binding sites and the effects of the cerebellar mutations on them is very similar and contrasts sharply with QNB binding. For example: (i) During development both DHA and muscimol binding have a phase of very rapid increase in receptor number between 15 and 20 days for muscimol (Figure 13) and 10 and 15 days for DHA (Figure 16). These time intervals coincide with periods of synapse formation on granule cell dendrites and sharp increases in binding may be related to synaptogenic events. No similar sharp increase was seen for QNB binding, which may indicate that these receptors are not directly associated with synapses. (ii) The change in DHA and muscimol binding relative to controls in the cerebellar mutants at 20 days takes the form, rl>wv>sg (Table 9). However for QNB binding the order is different rl>sg=wv. This suggests that the ways in which these mutations affect DHA and muscimol binding may be different for QNB binding. QNB binding is also less affected by these mutations than either DHA or muscimol binding; this despite the finding that muscarinic receptors are located in the molecular layer (Rotter et al. 1979b), which is drastically altered by the lack of parallel fibres (Sidman, 1968). These findings may be a further indication that QNB binding sites are not directly associated with synapses, and are thus not as markedly affected as DHA and muscimol binding (which, it is suggested, are located at synapses) by the synaptic reorganisation that occur in these mutants.

3.5 Future directions

Although the data presented in this chapter give some idea of the way in which receptors develop in relation to neuronal input and the way in which this relationship is affected by the cerebellar mutations, a number of questions remain unanswered. Some of these questions and possible solutions are outlined below.

The results of the muscimol binding study indicate that a large number of GABA receptors are localised on granule cells. However one cannot be certain that the reduction in muscimol binding seen in the agranular mutants is not an indirect effect of granule cell loss (similar to that seen for DHA binding). This problem might be solved by a more critical evaluation of GABA receptor development in the nervous mutant (Sidman & Green,1970) or another Purkinje cell-depleted mutant such as the Purkinje cell degeneration mutant (Mullen et al.1976). In both of these mutants the Purkinje cells die in the later stages of cerebellar development and the majority of the other neuronal cell types remain unaffected (Sidman & Green, 1970; Landis & Mullen, 1978). If a significant reduction in binding relative to controls was not observed over this period of Purkinje cell loss,then the assumption made by Olsen & Mikoshiba (1978) that GABA receptors on the Purkinje neurons make up only a small fraction of cerebellar GABA receptors might be justified.

The QNB binding results do not provide any direct evidence concerning the localisation of these receptors. Rotter et al. (1979b) have suggested that these receptors might be on climbing fibres. This possibility could be eliminated by the use of

3-acetylpyridine. This compound results in the death of climbing fibres (Desclin, 1974) and an examination of QNB binding in animals thus treated might reveal whether QNB receptors were located on these fibres.

As mentioned in Chapter 2 a method has been developed in this lab for the production of rat cerebellar cultures (Currie et al. 1979). Recently the culture conditions have been modified, allowing the successful culturing of mouse cerebellar neurons (Pigott & Dutton, unpublished). A number of workers have examined (^{3}H) muscimol and (^{3}H) QNB binding in neuronal cultures (De Feudis et al. 1979b; Dudai & Yavin, 1978; Siman & Klein, 1979). In the case of (^{3}H) QNB such studies have provided useful information about receptor regulation and neurotransmission. For example Siman & Klein (1979) showed that the muscarinic receptors of cultured chick cerebral cells were reduced in number by the presence of cholinomimetics, while the muscarinic blocker atropine, brought about an increase. From these data these workers were also able to estimate the turnover time of these receptors. It is hoped that similar information might be gained for GABA and β-adrenergic receptors using the cerebellar cultures. One preliminary study has been performed on cultured mouse cerebellar neurons. In that study (^{3}H) QNB binding was shown to increase four-fold over the first two weeks in vitro (East & Dutton, unpublished), which suggests that these receptors are situated on cerebellar cells and not climbing fibres.

Autoradiographic procedures for the localisation of (^{3}H) muscimol

have been published (Chan-Palay 1978) and a method is available for the localisation of muscarinic receptors using (^{3}H) propylbenzilylcholine mustard (Rotter et al. 1979a) The adaptation of such techniques to examine receptors in culture might give a better idea of the precise cellular location of these receptors. The histofluorescence technique of Melamed et al. (1976) could also be used to visualise β-adrenoceptors in culture.

The ultimate aim of such studies is to examine not only the way in which receptor populations are modified by interaction with neurotransmitters and their analogues, but in addition, the way in which receptors (both number and distribution) of a particular cell type are influenced by the presence of other cells. Such questions can only be answered using cultured cells.

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