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16. In each section studied, the tectal layers were defined and grains were counted continuously from the connective tissue to the periventricular nuclei. Four sections were counted for each fish. The number of grains counted in each tectal region was divided by the cross-sectional area examined, density values obtained from all regions were summed for each brain, and the percentage of the total density that each layer represented was calculated. This method normalized the data for variations in the total radioactivity reaching the tectum in different fish.

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A Mutation Influencing the Transportation of Manganese, L-Dopa, and L-Tryptophan

Abstract. *Transportation of manganese,* L-*dopa, and* L-*tryptophan was slower through the tissues of intact pallid mice than through those of black C57Bl/6J mice, presumably because of the influence of the gene* pallid.

Since both circumstantial (*1*) and direct evidence (*2*) indicate links between the metabolism of manganese and that of biogenic amines, the search for a fundamental connection is relevant to studies of parkinsonism (*1*) and chronic manganese poisoning (*3*). Such a connection would be one reflected by a well-defined gene. We studied the effects of the mutant gene *pallid* on the transportation of manganese and of L-dopa (L-dihydroxyphenylalanine) into the brain for the following reasons: (i) a neurological syndrome of pallid mice (*4*) was prevented in offspring by feeding large amounts of manganese to mothers during pregnancy (*5*); (ii) pallid mice seemed insensitive to the cerebral effects of L-dopa, a precursor of catecholamines (*6*). (iii) In contrast, black C57Bl/6J mice, which do not have the gene *pallid*, had shown neither unexpected manganese requirements nor unusual responses to L-dopa.

Male pallid mice were obtained by first crossing pallid (*pa/pa*) females to C57Bl/6J (*Pa/Pa*) males and then backcrossing the F_1 males (*Pa/pa*) to pallid (*pa/pa*) females because inbreeding of pallid mice reduced their viability. Control black (*Pa/Pa*) males were obtained from the C57Bl/6J stock. All mice were reared on Purina Laboratory Chow unless specified otherwise.

Radioisotopes of manganese have been injected intraperitoneally into mice consuming Purina; they accumulate preferentially in the liver, disappear thereafter from that organ and from the intact body at fairly rapid, characteristic rates (*7–10*). On diets deficient in manganese, normal animals had shown a slow rate of loss of intraperitoneally injected radioactive manganese ($^{54}Mn^{2+}$) from the intact body and a retention of the tracer in the liver so that the remainder of the body received relatively less isotope (*7, 8*). If pallid mice distribute and eliminate ^{54}Mn at slower rates than do black mice, this should be demonstrable with a variety of manganese intakes. Therefore, pallid and black mice on either a low manganese diet (milk, 0.025 μg of Mn per milliliter) or a high manganese diet (Purina Laboratory Chow, 30 μg of Mn per gram) were compared after receiving carrier-free $^{54}Mn^{2+}$ intraperitoneally. The intact, living pallid mice showed a significantly slower rate of loss of ^{54}Mn from the whole body under both diets (Table 1). Upon removal of the abdominal viscera and the brain, the remaining carcasses of pallid mice had lower concentrations of ^{54}Mn tracer than did those of black mice, on both stock and milk diets. The reverse was true for the livers, as shown by the ratios of ^{54}Mn in the carcass to that in the liver (Table 1). Thus, the pallid mice exhibited the signs of slower manganese transportation found in manganese deficiency (*7, 8*).

An accepted criterion of manganese deficiency is a decreased concentration of natural metal (^{55}Mn) in tissues. Selected tissues from animals fed Purina were analyzed by a nondestructive modification of neutron activation analysis, after extensive calibration with the destructive method (*11*). Relative manganese deficiency was pro-

Table 1. Whole body loss and organ distribution of $^{54}Mn^{2+}$. The half-time of the whole-body loss of ^{54}Mn was calculated in each mouse for the first 7 days after isotope injection (*7*); the animals were killed between days 9 and 7. Parentheses indicate numbers of animals. Results are expressed as means ± standard error. Student's *t*-tests were applied only on corresponding pallid and black mice. Differences between black and pallid mice were minimized with high intakes of $^{55}Mn^{2+}$.

Diet	Half-time (days)		Ratio of carcass to liver concentration (count/min)	
	Black	Pallid	Black	Pallid
Milk (27 days)	6.1 ± 0.20 (12)	8.2 ± 0.33 (12)*	6.7 ± 0.98 (3)	3.6 ± 0.44 (4)*
Purina (9 days)	4.1 ± 0.16 (8)	7.1 ± 0.31 (9)*	6.5 ± 0.98 (5)	2.4 ± 0.14 (5)*
Milk + $^{55}Mn^{2+}$ (55 μg/ml, 27 days)	4.1 ± 0.10 (3)	4.8 ± 0.10 (3)†	18.6 ± 0.50 (3)	15.7 ± 1.01 (3)†
Purina + $^{55}Mn^{2+}$ (180 μg/g, 9 days)	3.9 ± 0.24 (4)	6.6 ± 0.31 (4)*	7.4 ± 0.94 (3)	5.1 ± 0.27 (4)†
Purina + $^{55}Mn^{2+}$ (0.2 mg, i.p., daily, 9 days)	1.8 ± 0.12 (5)	1.8 ± 0.13 (4)	20.0 ± 2.42 (5)	25.4 ± 6.45 (3)

* $P < .01$; † $P < .05$.

nounced in bones, slight but definite in brains, and absent in abdominal viscera of adult pallid as compared to black mice (Table 2). Analyses of bones from heterozygous mice yielded intermediate values. Adult pallid mice thus had manganese-deficient tissues despite ample concentrations of manganese in the liver.

A precedent for such manganese deficiency in the midst of plenty had been found in patients with active rheumatoid arthritis (*12*). A biological meaning of the manganese deficiency of the pallid mice remained to be determined. Since manganese deprivation can increase the fragility and decrease the density of bone (*13*), we looked for these changes in bones.

Tensile strength was determined in bones dried at 85°C; we placed the distal 3 mm of the bone over the jaws of an open vise, suspended a pan in the middle, and loaded the pan with 10-g weights until the bone broke. The tested bones of pallid mice were significantly more fragile than those of the black ones (Table 2), and their density and nitrogen concentration were slightly, but significantly, lower. The tensile strength of bones from heterozygous mice was intermediate. The manganese-deficient bones of pallid mice thus had functional changes.

Since low manganese concentration in bone was an indication of functional changes in the bone, such a reduced concentration in the brain might indicate changes in some function of the brain.

Gastric intubation of L-dopa (Nutritional Biochemicals, 8 mg per gram of body weight) in 15 black mice induced salivation, piloerection, panting, hyperactivity, stereotyped movements, corkscrew tails, red nozzles, jumping, imbalance while walking, and, in some, convulsions. Six of these mice died. In sharp contrast, the same dose barely induced a reaction, and caused no deaths, among 15 pallid mice. L-Dopa was therefore given orally to 4 pallid mice in doses of 12 mg/g, and then to 15 in doses of 16 mg/g. Two deaths occurred among the latter, but cerebral manifestations remained minor, a suggestion that L-dopa was absorbed at a slower rate in pallid mice.

To determine whether this slow transportation was limited to crossing the intestine, L-dopa was administered intraperitoneally. The manifestations were graded in each animal as 0, absent; 1, slight; 2, moderate; and 3, marked. A sum of 30 indicated maximal effects and 0, no effects. Six pallid and six black mice were given L-dopa intraperitoneally (0.4 mg/g), and were scored every 10 to 15 minutes. The mean and standard error of the maximal scores was 7.3 ± 0.9 for pallid, and 15.2 ± 0.9 for black mice ($P < .01$). With nine pallid and nine black mice receiving 1.0 mg of L-dopa per gram of body weight intraperitoneally, the scores were respectively, 10.8 ± 1.7 and 22.7 ± 2.0 ($P < .01$). Scores of heterozygous animals were intermediate. L-Dopa appeared to be reaching the brain from the peritoneum more slowly in pallid than in black mice, as judged from the outward cerebral manifestations.

Table 2. Differences between black and pallid mice consuming Purina. Parentheses indicate number of animals. Results are expressed as means ± standard error.

Characteristics	Black	Pallid
Manganese (μg/g)		
Liver	3.50 ± 0.30 (5)	3.80 ± 0.20 (5)
Kidney	5.10 ± 0.30 (7)	5.60 ± 0.20 (7)
Brain*	1.52 ± 0.06 (13)	1.35 ± 0.04 (13)
Bone†	1.21 ± 0.06 (16)	0.74 ± 0.02 (20)
Tensile strength (g)		
Femur*	1612 ± 66 (19)	1386 ± 84 (8)
Humerus†	1000 ± 40 (18)	704 ± 29 (8)
Specific gravity*		
Femur	3.78 ± 0.11 (8)	3.35 ± 0.12 (8)
Nitrogen (mg/g)†		
Femur	2.83 ± 0.03 (7)	2.64 ± 0.05 (8)

* $P < .05$; † $P < .01$.

Cerebral effects of L-dopa have been correlated with cerebral concentrations of dopa and of dopamine (*6*). Whole brains were therefore analyzed 30 minutes after the intraperitoneal injection of water or L-dopa (0.4 mg/g). The results (Table 3) indicated that L-dopa had increased the concentrations of both dopa and dopamine in brain to a significantly lesser degree in pallid than in black mice.

L-Dopa is a large neutral amino acid whose transportation has similarities to that of other such amino acids (*1*). Tryptophan, the precursor of cerebral serotonin, is also a large neutral amino acid (*1*). Table 3 shows analyses for serotonin in animals receiving water or L-tryptophan (Nutritional Biochemicals, 0.4 or 0.8 mg/g) intraperitoneally, 30 minutes before being killed. The lower dose of L-tryptophan generated significantly less cerebral serotonin in pallid than in black mice, indicating that its transportation from the peritoneum to the brain was also slower in pallid mice.

Some of the large neutral amino acids, including those tested, are precursors of melanins; melanin granules are very rich in manganese (*14*). The melanosomes studied had high manganese uptakes, rivaling those of mitochondria (*9, 15*). These considerations agree with our findings in view of the contrast in pigmentation between pallid and black mice.

Taken together, our results indicate that a single gene can influence the transportation of a metal and of two amino acids in the same direction, provided that pallid and black mice differ only by a single gene (*4*). Phenylalanine, which is instrumental in phenylpyruvic oligophrenia, was not tested.

Concomitant distribution of large neutral amino acids and of manganese can occur either with or without the formation of corresponding chelates.

Table 3. Brain amine concentrations. The animals, consuming Purina, were killed at midday (11:30 a.m. to 12:30 p.m.). Parentheses indicate numbers of animals. Results are expressed as means ± standard error. Subtraction of the control values increased the differences between black and pallid mice; the high dose of tryptophan minimized these differences.

Injection	Animals	Concentration (μg per brain)
	Dopa	
Water	Black (5)	0.08 ± 0.005
	Pallid (5)	0.08 ± 0.010
	Dopamine	
Water	Black (5)	0.49 ± 0.007
	Pallid (5)	0.48 ± 0.013
	Dopa	
L-Dopa (0.4 mg/g)	Black (6)	1.16 ± 0.133
	Pallid (6)	0.77 ± 0.100*
	Dopamine	
L-Dopa (0.4 mg/g)	Black (6)	2.38 ± 0.119
	Pallid (6)	1.25 ± 0.142†
	Serotonin	
Water	Black (6)	0.21 ± 0.016‡
	Pallid (5)	0.19 ± 0.008
	Serotonin	
L-Tryptophan (0.4 mg/g)	Black (5)	0.29 ± 0.016
	Pallid (5)	0.22 ± 0.015†
	Serotonin	
L-Tryptophan (0.8 mg/g)	Black (4)	0.39 ± 0.029
	Pallid (3)	0.33 ± 0.047

* $P < .05$ (pallid versus black mice); † $P < .01$ (pallid versus black mice); ‡ $P < .01$ (tryptophan-treated versus control black mice).

Other amino acids, however, bind manganese (*16*). That L-dopa might conform to this precedent is suggested by the increased concentration of ^{54}Mn (*17*) and of ^{55}Mn (*2*) in livers of mice consuming L-dopa.

Such a connection was also implied in man by a drop of blood manganese (^{55}Mn) while the patients' parkinsonism was improved with dopa (*18*) (Fig. 2). This was, however, not an indicator of responsiveness to the drug (*19*). When ranking patients as to their responses to L-dopa, one might rank them also with some simplified version of a technique utilizing ^{54}Mn (*3, 12*) and seek correlations between the two. The individual variance is so great among patients with parkinsonism and chronic manganese poisoning (*1*) that the genetic differences encountered by others (*20*) might indicate divergent genetic susceptibilities among members of these populations. One cannot test healthy blood relatives with chronically administered drugs. It remains to be determined, however, whether a genetic marker can be developed by scanning these relatives for absorption or distribution of a harmless isotope (*3, 12*).

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Branching of Central Neurons: Intracellular Cobalt Injection for Light and Electron Microscopy

Abstract. *Cobalt chloride can be injected into an identified nerve cell body in an insect ganglion and reacted with ammonium sulfide to stain the soma and its branches with a black precipitate. The stained cell body and its branches throughout the neuropil are visible in both the light and electron microscope. In whole mount preparations, the resolution of neurites within the neuropil is of a quality that permits the comparison of branching patterns between cells and during various functional states.*

The specific form of a behavioral act generated by a population of neurons may be critically determined by the pattern of connections between units in the system. It is therefore essential in analyzing the cellular basis of behavior to be able to identify the individual neurons involved and to visualize the geometry of their interacting, branched processes (neurites). One of the most useful techniques for observing individual neurons has been the Golgi method and its variations. This has been a powerful tool because a small proportion of a neuronal population is stained and those neurons that have been stained are filled entirely. Although this method gives much cellular detail and has been extended from the light microscopic to the ultrastructural level (*1*), it has the major disadvantage that neurons are stained more or less randomly and that the quality of the results can be capricious.

The correlation of neural structure and function was advanced with the technique of intracellular injection of the fluorescent dye Procion Yellow (*2*). With this and other related dyes, neurites deep within the neuropil were stained in cells whose function had been determined by intracellular stimulation and recording. There are, however, some disadvantages with the method. The Procion dye is not electron-opaque, a fact that precludes observations at the ultrastructural level. Therefore, the precise determination of synaptic contacts on the neurites of the identified cells has not been possible. We have also found it difficult to observe the detailed branching pattern of nerve processes in whole mounts of the insect central nervous system with the use of Procion Yellow. Indeed, it has been necessary in the insect, as well as in most other invertebrate preparations, to reconstruct the branching pattern of neurites from serial sections. Even in this situation, we feel that only the major branches are clearly seen and the finer processes are not adequately resolved. We have developed an intracellular injection technique for neurons which permits the direct visualization of finely branched neurites in whole mounts viewed with bright-field illumination. Processes approaching 1 μm diameter are resolved. The dye is also electron-opaque. This allows ultrastructural observation of the injected cell and its relation to other elements of the neuropil.

Our method uses the reaction of cobaltous chloride with ammonium sulfide to form a black precipitate within the neuron. It should also be possible to use the chlorides of copper, iron, or nickel, all of which are soluble and are precipitated by ammonium sulfide. Neurons were filled with the cobalt solution by either the pressure injection technique of Remler *et al.* (*3*) or by iontophoresis. For pressure injection, glass micropipettes (tip diameter, 1 to 2 μm) were filled with a 1*M* solution of cobalt chloride containing 0.4 percent Procion Navy Blue H3RS. The Procion dye was added so that filling of the neuron could be observed as cobalt chloride is not sufficiently colored to be visible when the dye is entering the cell body. When the cell body was injected iontophoretically a 1 to 50 m*M* solution of cobalt chloride was used. The Procion dye is not re-