A DEVELOPMENTAL TIME SCALE FOR POSTNATAL CHANGES IN BRAIN AND BEHAVIOR OF B6D2F$_2$ MICE

By Douglas Wahlsten

Wahlsten, D. A developmental time scale for postnatal changes in brain and behavior of B6D2F$_2$ mice. Brain Research, 1974, 72, 251-264.

Summary:
A time scale for postnatal development was devised by testing separate litters of mice on 10 consecutive days (27.0-36.0 gestation days, or 8.5-17.5 days after birth) for behavioral abilities, body weight, brain weight, myelination of 80 fiber tracts and thickness of the external granular layer of the cerebellum (EGL). Although body and brain weights showed large variance within a litter or unstable changes across days, the other measures of brain and behavior underwent precise, monotonic changes with age. A time scale employing measures of reflexes, myelination and EGL thickness was presented which could be used to predict accurately the developmental age of a mouse. Detailed data were presented which revealed a general caudal—rostral gradient for brain maturation and a rostral—caudal trend for reflex ontogeny.

Article:

INTRODUCTION

The goal of the present research has been to establish a time scale for postnatal development in the laboratory mouse which can subsequently be employed to assess quantitatively the degree of ontogenetic retardation or acceleration imposed by either genetic or environmental variables. Towards this end, a wide range of neural and behavioral phenotypes has been measured at several times following birth; and equations have been derived in order to predict developmental age of an individual from the array of its phenotypes. For the purposes of this time scale, it was considered imperative to use several measures of the developing organism in order that the scale not be confined to a small subset of neural functions which might respond atypically to experimental manipulations. Measures of brain and behavior were sought which reflected the functioning of a wide range of brain regions and behavioral abilities. Although developmental variation in restricted facets of brain and behavior could of course be examined at the same time, the primary intent was to calculate the developmental age of the whole, functioning mouse with reference to a standardized population. Such a time scale should then serve to advance comparisons of genetic and environmental effects which are of growing interest to a variety of researchers in the developmental area[2].

All of the phenotypes included in the present study have been observed previously by a variety of researchers. Relevant phenotypes studied during the pre-weaning phase of postnatal ontogeny of the mouse have included simple reflexes and external morphological traits[5,11,19], brain weight[7,9], myelination of several fiber tracts[8,18], and the thickness of the external granular layer of the cerebellum[10]. These traits were selected for further research from a very large set of possible phenotypes primarily because they showed reliable, monotonic changes with age during the times of interest and because they were measurable with relatively little technological complication.

Several limitations of these previous studies precluded their direct application to the construction of a single time scale. For instance, the genotypes of mice varied widely between studies. Although genotype has sometimes been regarded as a minor influence upon developmental rate (*e.g.*, Fox[5], p. 238; Rugh[13], p. 1), contrary indications have been noted[11,17]. Furthermore, the environmental conditions and measurement criteria

were sufficiently dissimilar or vague in these studies as to require replication of the research at the very least.

Hence the need was apparent for a single study which evaluated all phenotypes in a precisely-defined and replicable genetic population of mice. In order to derive a time scale of maximum sensitivity, a developmental epoch in which all relevant phenotypes showed rapid change was sought. From the above studies as well as pilot research, times for a daily series of tests were chosen beginning shortly after the onset of myelination (about 8 days after birth) and extending to the onset of the 'popcorn' stage of hyperreactivity (about 17 days after birth). Separate litters were tested at each age to eliminate possible effects of repeated testing on developmental rate.

METHODS

Subjects

Parent mice were of the B6D2F_1/J strain from the Jackson Laboratory, Bar Harbor, Maine; they were the F_1 offspring of a cross between the isogenic strains C57B146J (female) and DBA/2J (male). The B6D2F_1 mice were chosen because they were genetically uniform as well as excellent breeders. As a result, their offspring represented an F_2 population (termed B6D2F_2) with genetic heterogeneity and outstanding viability. Although genetic variation undoubtedly contributed to phenotypic variance, this shortcoming was ameliorated by the superior care provided by an as opposed to an inbred mother; the larger litters produced by F_1 mothers also yielded a more accurate measure of the mean phenotypic value for the litter.

Breeding

One male and two naive female B6D2F_1/J mice were mated per standard mouse cage at 60 days of age. Routine examination was conducted for the presence of a vaginal plug[16] at least twice each day, and times of plugs were recorded. Chronological age of the offspring was taken from the midpoint of the interval between detection of a plug and the previous plug check; the midpoint was regarded as a gestation age of 0 days, 0 h. The male mouse was removed when both females had been plugged. A total of 10 females were impregnated and delivered normal litters. Each female was placed into a clean cage at least 1 day before birth, and she was left undisturbed until her litter was tested. All mice were maintained with free access to water and dry lab chow under a 12 h light-12 h dark schedule.

Testing

Since gestation length itself can vary as a function of both litter size and genotype[12], postnatal testing ages were calculated from the onset of gestation instead of from birth. One entire litter was tested each day from 27.0 to 36.0 days gestation age (about 8.5-17.5 days after birth); actual testing was within 2 h of the nominal time in all cases. Which litter was tested at which age was determined randomly.

At the appropriate time a mother and her litter were brought to a lighted testing room, and each young mouse was subjected to the same order of behavioral tests, weighing, anesthetization with chloroform vapor, and intracardiac perfusion with physiological saline followed by 10% formalin—saline. All mice within a litter were tested sequentially and as rapidly as was commensurate with accuracy; a litter of 10 mice required about 1.5 h to test.

Behavioral tests. The battery of behavioral tests was adapted from Fox[5] with a few additions and deletions. Each mouse was assigned a score on each test ranging from 0 to 1.0; a score of 0 indicated that no response was present at all, 1.0 indicated that the response was present in the mature or adult form, and numbers between 0 and 1.0 indicated the relative similarity of the response to the fully mature form. Detailed criteria for assignment of fractional scores are available from the author upon request.

Tests in the order administered and a brief description of each were as follows.

(1) Righting reflex. Does subject (S) return rapidly to its feet when placed on its back?

(2) Cliff aversion. Does S withdraw from the edge of a flat surface when its snout and forepaws are placed over the precipice?

(3) Forelimb grasp reflex. Does S grasp strongly the barrel of an 18-gauge needle when it is touched to the palm of each forepaw?

(4) Hindlimb grasp reflex. Same as forelimb test.

(5) Vibrissa placing reflex. Does S place its forepaw onto a cotton swab which is stroked across its vibrissae?

(6) Ears open. Are the auditory canals fully open?

(7) Ear twitch response. Is S capable of twitching its ear in response to a gentle puff of air or a gentle touch with a cotton swab?

(8) Level screen test, Can S hold onto a piece of 288-mesh aluminum screen when it is dragged across it horizontally by the tail?

(9) Vertical screen test. Can S hold onto the screen when it is placed in a vertical position?

(10) Screen climbing test. Can S climb up the vertical screen using both fore- and hindlimbs?

(11) Pole grasp. Can S grasp the shaft (2.5 mm) of a cotton swab firmly with both fore- and hindpaws?

(12) Forelimb stick grasp. Can S grip firmly a 9.5-mm-wide wooden stick with the forepaws ?

(13) Hindlimb stick grasp. Same as forelimb stick grasp.

(14) Eyes open. Are both eyes fully open?

(15) Visual placing reflex. Does S extend its forelimbs when it is lowered rapidly towards a flat surface?

(16) Auditory startle response. Does 5 show a whole-body startle response when a loud snap of the fingers occurs less than 6 in. away?

(17) Popcorn behavior. Does S show exaggerated jumping and running behavior in response to either a blast of compressed Freon gas or a gentle puff of the experimenter's breath?

Histology. Following intracardiac perfusion with formalin, the brain of each S was extracted from the skull and was weighed to the nearest milligram after at least one further week in formalin. Each brain was then encased in 10 % gelatin and stored in buffered formalin until sectioning. Serial sagittal sections were cut with a freezing microtome at a thickness of 25 µm, saving every fourth and fifth section. Alternate sections were subsequently mounted on gelatinized slides and stained with cresyl violet for Nissl substance and Sudan Black B forphoopholipids in myelin sheaths. the parameters for the Sudan stain were as follows : (1) 2 min in 70% ethanol; (2) 7 min in Sudan Black B; 1 % in 70% ethanol; (3) differentiate in 70% ethanol until background is nearly clear; (4) continue differentiation in 50% ethanol until background appears clear to the unaided eye; (5) rinse in several changes of distilled water; (6) mount and coverslip with Hydramount. Ten slides were run through the stain simultaneously, one from each of the 10 litters.

The degree of myelin staining was assessed within 10 days of staining by viewing sections at × 40 magnification, using the atlas of the mouse brain by Sidman *et al.*[15] or identification of the fiber tracts listed in

Table II. Where a tract was represented in both halves of the brain, such as lemniscus lateralis, a score was assigned based upon taming intensity of both halves. The scores and criteria for assignment are given below :

0 — No myelin staining is evident.
1 — Staining is faint, but the tract can definitely be distinguished.
2 — The tract is clearly seen, but the staining is not intense.
3 — The tract is clearly, intensely stained as it is in the adult brain.

All viewing was done blind insofar as the experimenter did not know the gestation age of the brain at the time of evaluation.

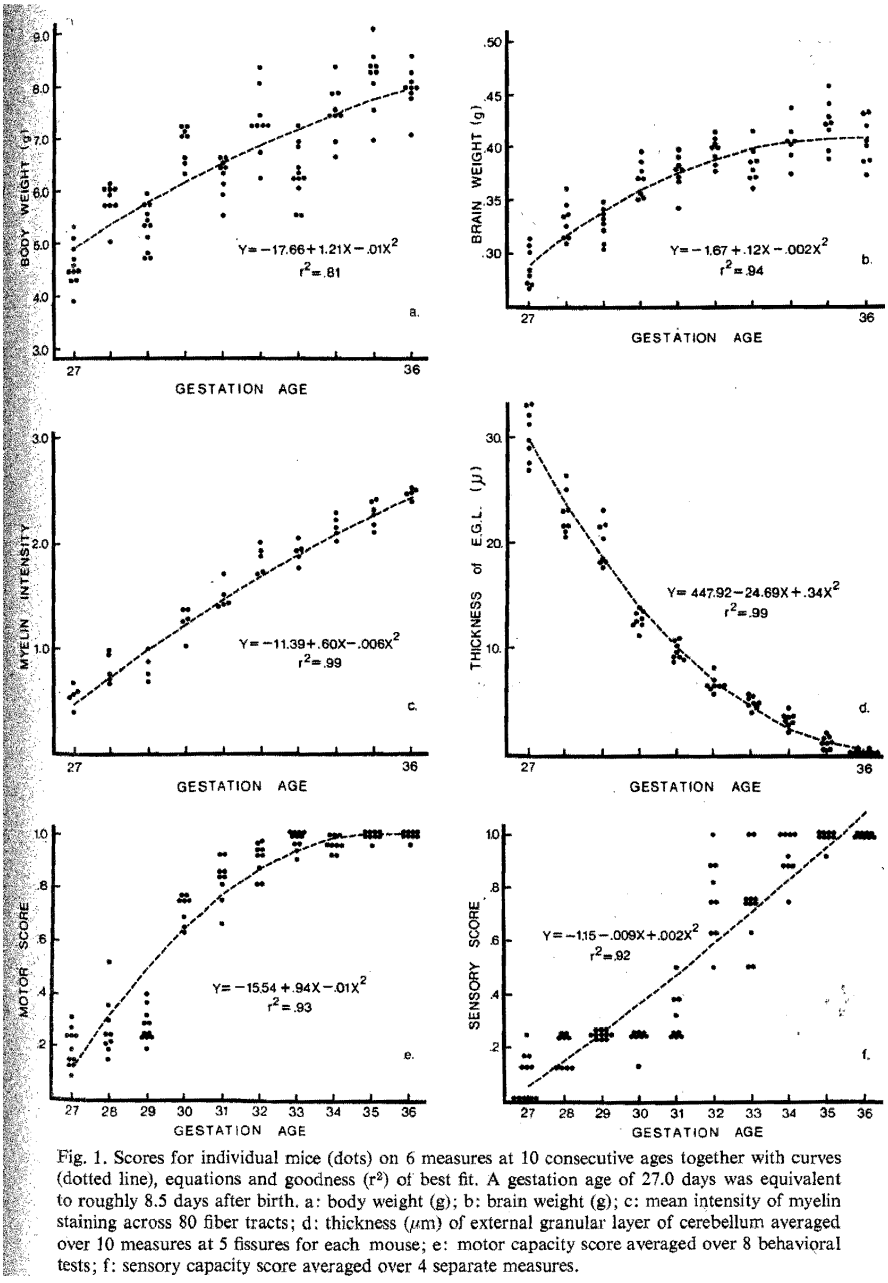

Fig. 1. Scores for individual mice (dots) on 6 measures at 10 consecutive ages together with curves (dotted line), equations and goodness (r^2) of best fit. A gestation age of 27.0 days was equivalent to roughly 8.5 days after birth. a: body weight (g); b: brain weight (g); c: mean intensity of myelin staining across 80 fiber tracts; d: thickness (μm) of external granular layer of cerebellum averaged over 10 measures at 5 fissures for each mouse; e: motor capacity score averaged over 8 behavioral tests; f: sensory capacity score averaged over 4 separate measures.

Thickness of the external granular layer (EGL) of the cerebellum was measured with an ocular microscale, viewing at × 1000 under oil immersion. All measures were taken from the section closest to the mid-sagittal plane in the Nissl-stained series. Thickness of the proliferative zone perpendicular to the fissure was measured on both surfaces of the fissure at its intermediate level for each of 5 fissures (fissura posterolateralis, fissura secunda, fissura prima, fissura preculminata, fissura precentralis). Hence, the score for each S was the arithmetic mean of 10 separate measures of thickness.

All behavioral testing and viewing of myelination and external granular layer were done by the author.

RESULTS

All 10 females were plugged within 10 days, their gestation periods ranged from 18.1 to 18.6 days, and their litter sizes ranged from 8 to 11, thus corroborating previous experience with their excellent breeding qualities.

All mice in each litter received the behavioral test battery and were weighed, but only the first 8 mice tested in each litter were perfused and processed histologically. Of these 8, all were evaluated for EGL thickness, but only 5 were viewed for myelin staining intensity.

TABLE I

MEAN SCORES ON EACH BEHAVIORAL TEST AT EACH CHRONOLOGICAL AGE

Test	*Chronological age*									
	27	*28*	*29*	*30*	*31*	*32*	*33*	*34*	*35*	*36*
Righting reflex	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Cliff aversion	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Forelimb grasp reflex (M)*	0.82	0.90	0.89	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Hindlimb grasp reflex (M)	0	0	0	0	0.61	0.94	1.00	0.97	1.00	1.00
Vibrissa placing (S)**	0.36	0.78	1.00	0.94	1.00	1.00	0.91	0.89	1.00	1.00
Ears open	0	0	0	0.25	0.72	1.00	1.00	1.00	1.00	1.00
Ear twitch response	0	0	0	0.06	0.06	0.89	0.86	1.00	1.00	1.00
Level screen test (M)	0.18	0.13	0.06	1.00	0.86	1.00	0.97	1.00	1.00	1.00
Vertical screen test (M)	0.52	0.67	0.68	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Screen climbing test (M)	0.04	0.22	0.11	0.88	1.00	0.83	1.00	0.97	1.00	1.00
Pole grasp (M)	0.01	0.19	0.26	0.58	0.63	0.81	1.00	1.00	0.86	0.97
Forelimb stick grasp (M)	0.14	0.08	0.22	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Hindlimb stick grasp (M)	0	0	0.02	0.31	0.54	0.67	0.86	0.80	1.00	1.00
Eyes open (S)	0	0	0	0	0.26	1.00	1.00	1.00	1.00	1.00
Visual placing reflex (S)	0	0	0	0	0	0.67	0.18	0.80	0.97	1.00
Auditory startle (S)	0	0	0	0	0	0.37	0.86	1.00	1.00	1.00
Popcorn behavior	0	0	0	0.05	0.02	0	0.34	0.52	0.71	0.64
Mice in litter	11	9	11	8	9	9	11	9	9	9

* M indicates that the test was incorporated into the composite motor capacity score.
** S indicates similarly for the sensory capacity score.

Basic observations

Of the data collected for each mouse, individual values for body weight are shown in Fig. 1 a, and mean scores at each age are given for all behavioral tests in Table I. In general, a smooth, monotonic increase was observed for all variables. However, 4 variables were not used in subsequent composite scores or other analyses. Righting reflex and cliff aversion were quite mature at all ages tested, while ear opening and ear twitch were believed by the experimenter to be too unreliable and tainted with subjective criteria to be useful. The remaining 8 tests which reflected primarily the maturation of motor capacities, indicated in Table I, were averaged for each mouse to yield a composite motor capacity score, shown in Fig. le. Likewise, the 4 tests which reflected sensory maturation were averaged, and individual sensory capacity scores are shown in Fig. If. It can be seen that at 27 days gestation age mice of this cross are generally quite incapable of performing most adult responses at all, while by 36 days all patterns are relatively mature.

Brain weight and mean thickness of EGL are shown for each of 8 mice per age in Fig, lb and Fig. 1d. Again, a very regular change with age was observed. By 36 days brain weight was only about 50 mg less than that of adult mice of the same cross, and the EGL was virtually gone, with only an occasional round, compact cell observable at the surface of any of the 5 fissures.

The progress of myelination is presented for each fiber tract according to its region in the brain in Table II. Ages given are those at which the mean myelin intensity for a particular tract first exceeded the criterion of 0,5, 1.5 or 2.5 and did not fall below that criterion at more advanced ages. Since no fractional scores were assigned to individual brains, a tract which is indicated as beyond 0.5 at day 28 in Table II, for example, is one for which more than half the mice at that age were at intensity 1 or greater. Hence, age at 0.5 was the earliest point where any degree of myelination could be reliably detected, and age at 2.5 represented the time of approach toward adult staining intensity. Mean myelin scores for each brain region across days are portrayed in Fig. 2, while the mean score for all 80 tracts is shown for each mouse in Fig. lc. Not only was a monotonic increase in myelin

intensity generally found, but a caudal—rostral sequence of myelination was also evident.

Although all phenotypes exhibited a regular change with age, the variability of scores within a litter was relatively large for certain measures. Both motor and sensory scores undoubtedly varied widely at least in part because of inconsistencies introduced into the testing procedure by the human experimenter, but body and brain weights were measured very accurately and therefore must reflect genuine individual differences in the mass of nutrients incorporated into the growing mouse. On the other hand, mean myelin intensity and EGL thickness scores were highly similar within a litter, and very little overlap between litters differing in age by one or two days was observed.

Curves of best fit and calculation of developmental age

In order to compute developmental age from an array of phenotypes for an individual, it was necessary first to derive curves of best fit for scores of a standard population, in this case the B6D2F$_2$ mice. Given the generally smooth, monotonic relations portrayed in Fig, 1, curves of best fit were found for several phenotypes using quadratic regression. Fitting to the relation $Y = a_0 + a_1X + a_2X^2$ was useful because of its mathematical simplicity and because it made no assumptions about the natures of underlying processes such as are implicit in the normal ogive or other specific models, A satisfactory fit was obtainable using only the first and second powers, although a perfect fit was of course possible using a Taylor series with more terms.

TABLE II

CHRONOLOGICAL AGES AT WHICH EACH FIBRE TRACT* EXCEEDED SPECIFIED MEAN MYELIN INTENSITY SCORES

Structure	*Mean myelin intensity*		
	0.5	*1.5*	*2.5*
Spinal cord			
Tr. corticospinalis	27	31	33
Fibrae corticospinales	27	31	33
Funiculus dorsalis	**	27	30
Funiculus ventralis	**	27	30
Funiculus lateralis	27	29	31
Tr. spinalis n. trigemini	27	29	32
Cranial nerves			
R. n. hypoglossi	**	27	31
R. n. facialis, pars ascendens	27	28	32
R. n. facialis, pars descendens	**	27	30
Genu n. facialis	**	27	31
R. n. vestibulocochlearis	27	29	30
R. motoria n. trigemini	**	27	31
R. sensoria n. trigemini	**	27	30
R. n. trochlearis, pars intramedullaris	27	30	***
R. n. trochlearis, pars extramedullaris	**	27	30
R. n. oculomotorii	27	31	36
Tr. opticus	28	31	35
Tr. olfactorius lateralis	29	30	34
Rhombencephalon			
Tr. solitarius	27	30	***
Fibrae arcuatae internae	30	34	***
Fasciculus longitudinalis medialis	**	27	32
Tr. tectobulbaris	**	27	32
Tr. corticospinalis	27	29	32
Fibrae longitudinales pontis	27	32	***
Fibrae transversae pontis	30	31	34
Lemniscus medialis	27	29	***
Corpus trapezoideum	**	27	28
Tr. tectopontinus	28	31	***
Tr. olivocerebellaris	30	33	***
Tr. spinocerebellaris ventralis	27	28	31
Pedunculus cerebellaris superior	28	31	36
Pedunculus cerebellaris inferior	27	28	32
Pedunculus cerebellaris medius	27	30	34
Comm. cerebelli	27	29	32
Corpus medullare cerebelli	27	29	32
Laminae albae cerebelli	27	30	34
Mesencephalon			
D. pedunculorum cerebellarium superiorum	28	30	34
D. dorsalis tegmenti	29	30	34
Lemniscus trigeminalis	29	32	35
Comm. colliculorum anteriorum	30	32	***
Comm. colliculorum posteriorum	30	32	35
Fasciculus longitudinalis medialis	27	30	35
Lemniscus medialis	28	32	36

(continued on next page)

(Table II continued)

Structure	*Mean myelin intensity*		
	0.5	*1.5*	*2.5*
Crus cerebri	28	32	35
Tr. mesencephalicus n. trigemini	30	34	***
Tr. corticotectalis	31	36	***
Tr. tegmentalis centralis	28	31	***
Tr. spinotectalis	29	34	***
Brachium colliculi posterioris	29	32	36
Lemniscus lateralis	27	30	34
Diencephalon			
D. supramammillaris	32	36	***
Comm. posterior	28	30	***
Stria medullaris thalami	30	34	***
Fasciculus retroflexus	29	32	***
Fasciculus mammillothalamicus	32	35	***
Columna fornicis	32	***	***
Lemniscus medialis	31	33	***
Crus cerebri	29	32	36
Radiato inferior thalami	31	36	***
Radiato superior thalami	33	35	***
Radiato intermedia thalami	30	32	36
Brachium colliculi anterioris	31	34	***
Telencephalon			
Comm. anterior, pars anterior	32	35	***
Comm. anterior, pars posterior	34	***	***
Tr. olfactorius intermedius	31	33	***
Fornix	32	36	***
Fornix precommissuralis	33	***	***
Alveus hippocampi	32	36	***
Fimbria hippocampi	32	34	***
Genu corporis callosi	36	***	***
Truncus corporis callosi	33	***	***
Splenium corporis callosi	33	***	***
Forceps minor corporis callosi	31	34	***
Forceps major corporis callosi	31	34	***
Radiato corporis callosi	28	32	***
Cingulum	30	33	***
Capsula interna	28	32	36
Ansa lenticularis	33	36	***
Fibrae ganglii basalis	30	34	***
Comm. fornicis dorsalis	31	34	***

* Abbreviations used are as follows: Tr., tractus; R., radix; n., nervus; Comm., commissura; D., decussatio.

** Indicates that the tract was at least of intensity 1.5 at the youngest age tested and therefore that the age at which intensity 0.5 was achieved remains unknown.

*** Indicates that the tract never achieved the designated intensity within the first 36 days of life.

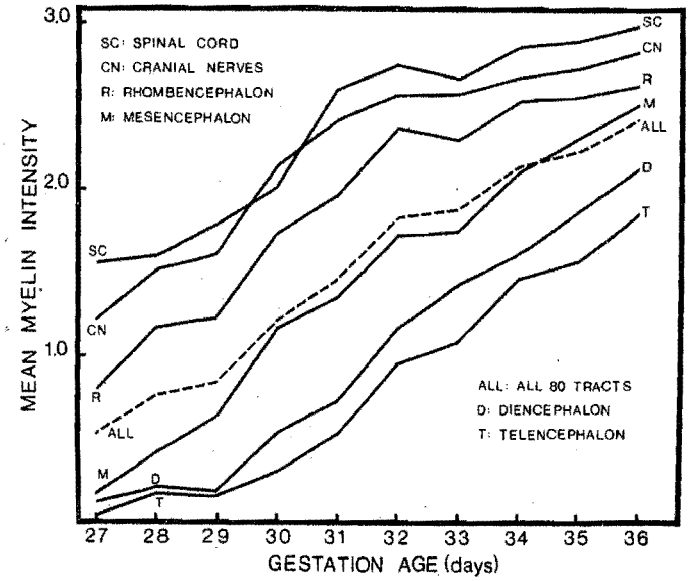

Fig. 2. Mean myelin staining intensity at 10 consecutive ages for 6 separate nervous system regions as well as for all 80 fiber tracts. The tracts comprising each region are listed in Table II.

Equations resulting from the quadratic regression of several phenotypes upon chronological age are expressed in Fig. 1 together with their corresponding curves of best fit and values for goodness of fit (r^2). The mean score for each litter was used to derive the curves, since it was desired to weight each litter equally, regardless of the number of mice in the group. A good fit to group means was evident for all phenotypes, while fits for myelin intensity and EGL thickness were extremely good. Curves for motor and sensory scores were clearly not satisfactory, since many scores were near the lower and upper limits at several ages, thus requiring a cumulative normal curve or similar device to obtain greater accuracy. It was found, however, that the abrupt jump in motor and sensory scores could be smoothed by taking as the phenotype the average of motor and sensory scores for each mouse. The resulting behavioral score was accurately fit ($r^2 = 0.96$) by the equation $Y = —8.34 + 0.466X - -0.006X^2$.

TABLE III

REGRESSION EQUATIONS USED TO PREDICT DEVELOPMENTAL AGE (Y) FROM PHENOTYPES (X)

Phenotype	*Regression equation*	*Goodness of fit (r^2)*
Body weight (g)	$Y = 24.35 — 0.35X + 0.21X^2$	0.81
Brain weight (g)	$Y = 51.76 — 188.36X + 356.19X^2$	0.90
Myelin intensity	$Y = 25.86 + 2.46X + 0.68X^2$	0.99
EGL thickness (μm)	$Y = 35.66 — 0.54X + 0.01X^2$	0.99
Motor capacity (M)	$Y = 30.33 — 14.22X + 18.65X^2$	0.91
Sensory capacity (S)	$Y = 26.96 + 8.89X — 0.83X^2$	0.92
Behavior (M/2 + S/2)	$Y = 26.93 + 3.98X + 4.12X^2$	0.96

Although a reasonably orderly change with age was evident in all phenotypes, it was clear (see Fig. 1) that certain measures showed a less consistent progression across days and more variability within a litter than did others; hence it was apparent that all measures would not be equally useful in calculating developmental age. In order to make clear this situation in terms of accuracy of prediction in fractions of a day, quadratic regression equations of chronological age upon each phenotype were derived (see Table III) using group means, The resulting equations were then used to compute the developmental age of each subject on the basis of each of its phenotypes. The resulting means and variances of predicted developmental age based upon each phenotype are given for each litter in Table IV.

TABLE IV

MEANS AND VARIANCES OF DEVELOPMENTAL AGES DERIVED FROM EACH PHENOTYPE AT EACH CHRONOLOGICAL AGE

Chron. age	*Body weight*		*Brain weight*		*Myelin*		*EGL*		*Behavior*	
	Mean	*Var.*	*Mean*	*Var.*	*Mean*	*Var.*	*Mean*	*Var.*	*Mean*	*Var.*
27	27.28	0.41	27.15	0.12	27.44	0.10	27.30	0.002	27.59	0.09
28	29.49	0.45	28.52	0.75	28.32	0.27	27.86	0.07	28.10	0.11
29	28.48	0.62	28.38	0.46	28.37	0.22	28.35	0.14	28.28	0.04
30	32.01	0.77	30.94	1.57	30.06	0.38	30.20	0.06	29.77	0.05
31	30.53	0.67	31.48	1.62	31.05	0.34	31.21	0.11	30.56	0.33
32	33.13	2.95	33.28	1.40	32.75	0.42	32.43	0.09	33.13	1.06
33	30.64	1.56	32.09	2.35	33.06	0.27	33.18	0.07	33.40	0.82
34	33.63	1.99	34.02	4.00	34.32	0.34	33.92	0.13	34.36	0.33
35	35.57	3.29	35.92	6.57	34.99	0.43	35.12	0.05	34.94	0.03
36	34.85	1.43	34.11	5.10	36.04	0.07	35.46	0.01	34.99	0.01

Whereas the precision of estimates based upon body weight and brain weight was unimpressive, variances of estimates from myelin intensity, EGL thickness, and the behavioral index were generally very small. It was therefore concluded that a satisfactory scale for developmental age could be established by taking the arithmetic mean of the ages predicted from the latter 3 variables. The outcome of this averaging procedure is shown in Table V based upon the 5 mice in each litter for which all measures were available.

Two important points emerged from this analysis. First, estimates of age corresponded closely with chronological age, as would be expected, and the standard errors of the estimates were sufficiently small to inspire confidence in the scale. Second, the variance of estimated age was frequently greater than the variance

of age derived from EGL thickness by itself (see Table IV). This was not too astounding, since the EGL measure was so closely ($r^2 = 0.99$) related to age in its own right. Averaging the 3 age estimates thus will likely increase the variance, but it is necessary for future application of the scale which must not rely solely upon one particular measure of the growing, complex system.

TABLE V

MEAN, VARIANCE AND STANDARD ERROR OF DEVELOPMENTAL AGE DERIVED FROM AVERAGE OF MYELIN, EGL AND BEHAVIORAL AGES AT EACH CHRONOLOGICAL AGE

	Chronological age									
	27	*28*	*29*	*30*	*31*	*32*	*33*	*34*	*35*	*36*
Mean age	27.46	28.10	28.28	29.97	30.98	32.62	33.28	34.12	34.98	35.49
Variance	0.045	0.097	0.036	0.049	0.027	0.056	0.184	0.127	0.063	0.009
Standard error	0.095	0.139	0.095	0.099	0.073	0.106	0.192	0.160	0.103	0.042
n*	5	5	4	5	5	5	5	5	6	5
Litter size	11	9	11	8	9	9	11	9	9	9

* n represents the number of mice from each litter for which all measures were available and upon which the developmental age was consequently based.

DISCUSSION

Although the time scale indeed predicted the developmental ages of litters from which it was derived in close agreement with their chronological ages, the fit was not perfect Errors of approximately one-half day resulted at ages 27, 29, 32 and 36 (Table V). The problem at age 36 was clearly that the EGL and behavior scores had reached an upper limit. Several possible explanations for errors at other ages exist, however. For one thing, the interval between successive plug checks ranged from 8 to 14 h, and hence the true gestational age could vary by nearly half a day. Furthermore, genetic differences within a litter might increase the variance in developmental age if genotype is in fact an important determinant of developmental rate. Taken together with errors inherent in the measurement devices themselves, especially those introduced by the experimenter's fallible judgment, these various perturbations in the time scale are at least as great as the observed deviations from perfection.

It is worthwhile at this point to compare the temporal phenotypic changes observed in this experiment to those seen by other researchers. Briefly, results were very similar to those previously reported for body weight[14], brain weight[7], reflex ontogeny and eye opening[5], and thickness of the external granular layer of the cerebellum[10], taking into account the fact that most previous studies used birth as the beginning of time; any discrepancies were on the order of a day or two at most. The schedule of myelination, on the other hand, disagreed in several respects with previous reports.

It is easy to imagine numerous factors which might influence the intensity of myelin staining in otherwise equally mature brains. Fixation procedures can certainly affect staining[3], while the affinity of a stain for certain myelin components or its discriminability from background staining and its inherent contrast can likewise affect a tract's visibility to a human observer. The strain of mouse employed might introduce a few days variation one way or the other, especially if gestation lengths differed. These factors may explain why Kelton and Rauch[8] observed that almost all tracts had begun myelination by 11 days after birth using fixation by immersion in formalin and staining with the Weigert—Pal method on mice heterozygous for the dilute-lethal (d^1) gene, while Uzman and Rumley[18], using no immediate fixation and the Klüver—Barrera stain on Swiss mice, concluded that 'up to 10 days of age the mouse brain was substantially free of myelin' (p. 179), except for a few midbrain structures; Kelton and Rauch observed myelination in corpus callosum by post-natal day 6 while Uzman and Rumley reported the beginnings at day 20. However, no such procedural variations can account for differences in the *order* in which structures begin myelination. In marked disagreement with the present study, Kelton and Rauch reported myelination of commissura anterior, pars anterior (day 5) *before* corpus trapezoideum (day 6) and late appearance of lemniscus lateralis (day 17) and radix nuclei oculomotorii (day 19), whereas the corresponding postnatal ages of appearance in the present study were about 14, 9, 9 and 9 days, respectively (Table II). Moreover, Kelton and Rauch did not even observe a general caudal—rostral sequence of myelination, in contrast with previous studies of myelination[6]. Until some intrepid anatomist undertakes a count of myelin lamellae in all these fiber tracts at several ages using electron microscopy, these conflicting results

will be difficult to resolve. For the present purposes, nonetheless, intracardiac perfusion with formalin and staining with Sudan Black B seem to be convenient and reliable methods for observing myelination at a gross level. Providing that the same methods are used in future studies, myelination should serve as a useful item in the developmental time scale.

The validity and utility of this time scale, of course, must be established by its future application to interesting developmental problems. The regression equations (Table III) should make possible the estimation of developmental age differences as small as one-half day (95 % confidence interval of 0.5 day) using only 10-15 mice per group. Assuming that an untreated $B6D2F_1 \times B6D2F_1$ control group is always included in the study for purposes of comparison, developmental age of any group may then be expressed in $B6D2F_2$-equivalent gestation days' with a specified confidence interval. It will hence become possible not only to assign precise magnitudes to temporal variations imposed by nutritiona1[1,4] and genetic[11] variables but also to compare the *relative* potencies of genetic and environmental influences upon the rate of maturation of mouse brain and behavior.

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