0270-6474/83/0311-2336$02.00/0
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Printed in U.S.A.
The Journal of Neuroscience
Vol. 3, No. 11, pp. 2336–2349
November 1983

QUANTITATIVE FEATURES OF SYNAPSE FORMATION IN THE FLY'S VISUAL SYSTEM

I. The Presynaptic Photoreceptor Terminal[1]

A. FRÖHLICH[2] and I. A. MEINERTZHAGEN

Department of Psychology, Life Sciences Centre, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4J1

Received November 16, 1982; Revised June 2, 1983; Accepted June 6, 1983

Abstract

Photoreceptors of the adult fly's compound eye each form a population of stereotyped output synapses distributed over the surface of their terminal. The formation of this class of afferent synapses during development has been followed from serial electron microscopy of the same eye region in four pupal and several adult stages, all of female *Musca domestica*. These synapses, or tetrads, have an invariant postsynaptic composition of four members and so may provide a model for multiple-contact synapses in general. In the adult fly the four postsynaptic elements of each synapse are contributed by two interneurons, L1 and L2, and, usually, two α processes of an amacrine cell. These postsynaptic elements assemble at individual developing synapses by selective sequential addition. Assembly starts with L1 or L2, subsequent elements of the final tetrad adding in all conceivable permutations, at least as fast as one per 7 hr. They rarely (only once) incorporate incorrect or supernumerary elements, however. The synaptic population as a whole was also sampled during development to analyze the possible factors determining the normal precision of the size of the adult population. The number of synapses per terminal increases gradually until 74% pupal development. Thereafter it decreases so that the final number of synapses in each receptor's population is the consequence of a net loss. Synapses enlarge with age, chiefly by incorporating new elements, but the loss of synaptic sites is only partially offset by the increase in size of those that remain. Throughout all stages examined in pupal and adult life, total synaptic area is linearly proportional to the surface area of the axon terminal. Thus, from the 74% pupal development stage onward, a population of many small synapses closely spaced, on average, over the terminal's surface transforms into one characteristic of the adult with progressively fewer, larger, more widely spaced synapses.

The formation of precisely constituted synaptic populations in the developing nervous system involves two types of phenomena. The first requires assembly of individual synaptic contacts between physiologically appropriate neurons, by mechanisms which presumably depend ultimately upon neuronal recognition (Barondes, 1976), and the second requires the regulation of the quantity or number of such contacts within any one functional class of synapse. These two features are quite separable aspects of synaptogenesis, the qualitative and the quantitative (Purves and Lichtman, 1980). The number of cellular participants at many synaptic contacts (either pre- or postsynaptic) often departs from the 1:1 relationship of most simple synapses analyzed to date. In certain types of specialized synapses (Shepherd, 1979), for example, where one neuron is presynaptic to clusters (dyads, triads, tetrads, etc.) of postsynaptic elements at a single synaptic junction, *qualitative aspects* of synaptogenesis assume special complexities because more than one neuron must explore and recognize each synaptic target. We refer to such arrangements as multiple-contact synapses (Meinertzhagen and Fröhlich, 1983). They are found widely and are perhaps the norm throughout invertebrate nervous systems (King, 1976; Muller and McMahan, 1976; Armett-Kibel et al., 1977; Watson and Burrows, 1982) as well as in specific areas of the verte-

[1] This work was supported by a grant from the Deutsche Forschungsgemeinschaft (to A. F.), National Science and Engineering Research Council Grant A-0065, and National Institutes of Health Grant EY-03592. We wish to thank M. G. Yoon for reading the manuscript, S. R. Shaw for detailed comments upon it, and Wenda Ellis for technical assistance.

[2] To whom correspondence should be addressed.

brate brain (Dowling and Boycott, 1966). Analysis of the *quantitative aspects* of synaptogenesis, on the other hand, is more straightforward in a system in which the innervation ratios established between pre- and postsynaptic cell populations undergo no developmental adjustments, such as result from cell death or synapse elimination (Purves and Lichtman, 1980). Developmental influences upon the number of synaptic contacts established can then be assigned to interactions between fixed pre- and postsynaptic partners. Neither the characteristic of multiple-contact synapse nor that of the fixity of cell partners generating a particular synapse class is, however, generally typical of the model motor synapses which have most frequently been used for studies on synaptogenesis.

An analysis of the quantitative aspects of synaptogenesis is reported here for the population of tetrad synapses which constitute the chief afferent transfer site of the photoreceptor terminals of the fly's compound eye. This class of synapse is one of two dozen or so within the circuitry of the first optic neuropil of the visual system, the lamina ganglionaris (Strausfeld and Campos-Ortega, 1977). Each photoreceptor terminal forms about 200 presynaptic contacts upon groups of four elements, derived exclusively from a fixed set of postsynaptic cells. These cells are four of the dozen or so occupants of the unit column, or cartridge, of the lamina. All cells are identified uniquely on morphological grounds. In serial electron micrographs, all of the synaptic contacts of a receptor terminal can be identified and counted. They may also be classified according to the number and composition of postsynaptic dendrites which may be traced through consecutive sections to their point of origin at an identifiable parent axon. The complement and cellular identity of elements communicating at each synapse, as well as the total number of such synapses per receptor terminal, are all highly predictable. Moreover, each contact has a high degree of geometrical stereotypy (Fröhlich and Meinertzhagen, 1982a; Nicol and Meinertzhagen, 1982a).

Synapse formation occurs after the large-scale events of neurite growth which bring intended pre- and postsynaptic neurons into juxtaposition. During the first half of pupal development, rapid innervation of the lamina by growth cones from photoreceptor axons is followed by the slow, complex redistribution of each growth cone to a particular location in a nearby cartridge (Meinertzhagen, 1973, 1977; Trujillo-Cenóz and Melamed, 1973; Hanson, in Kankel et al., 1980). Only after photoreceptor axons settle in their final positions during the last half of pupal development does synaptogenesis commence (Trujillo-Cenóz and Melamed, 1973; Fröhlich and Meinertzhagen, 1982a). Synaptogenesis proceeds by the growth and apposition of postsynaptic neurites to sites arrayed over the presynaptic receptor terminal membrane. This mode of growth is thus back to front to that occurring during synaptogenesis at many other systems. For example, during synapse formation at various neuromuscular junctions (e.g., Lømo and Jansen, 1980), it is the presynaptic neuron which grows to its postsynaptic target cell.

The fine-structural features of synapse formation in fly photoreceptors have been documented already for *Musca domestica* (Fröhlich and Meinertzhagen, 1982a). These include criteria for identifying synapses in the immature stages, the gradual increase with age in the number of elements contributing to the synapse, and the change in the size of individual synaptic contacts. These features form the basis for this report in which the growth and spacing of the synaptic population are quantified. Preliminary reports have appeared (Fröhlich and Meinertzhagen, 1979, 1982b).

Materials and Methods

Serial section electron microscopy has been utilized to examine females of the housefly *Musca domestica*. Details of the electron microscopic techniques and the rearing and developmental staging of animals have been provided elsewhere (Fröhlich and Meinertzhagen, 1982a). Developmental stages are defined by the age of the pupa, from which the adult emerges at eclosion. Starting with puparium formation (0%), each stage is identified by the proportion it has survived of the average time (about 120 hr) taken by sibling pupae until eclosion (100%). Animals are identified as percentage of pupal development stages, consistent with our prior usage (Fröhlich and Meinertzhagen, 1982a) and despite the fact that it is the development of the enclosed visual system of the adult which is being analyzed. This convention also avoids the need to identify the exact starting point of adult development within the earlier larval stages. Analyses are presented of electron micrograph series of up to 100 sections each, cut from one 3-week-old adult and three immature females (at stages of 74%, 81%, and 94% pupal development) as described previously (Fröhlich and Meinertzhagen, 1982a). For each stage at least two cartridges were so analyzed from a total of two specimens at a fixed (frontal, equatorial) eye region. In addition, for the analyses reported here, another three adult series (1-day, 4-day, and 10-day female) were similarly examined, but from a single cartridge in each case, for the number of photoreceptor synapses. A preliminary examination was also undertaken of a single series from a 62% pupal development stage. The overall depth of the lamina was measured from longitudinal sections. For this, semithin sections were cut, usually taken from the reimbedded 100-μm-thick section adjacent to that from which each particular series had been cut. For the series previously reported, the surface area and volume of the presynaptic photoreceptor terminals were computed from the product of section thickness and, respectively, the mean perimeter or area of photoreceptor terminal profiles measured in consecutive micrographs with the aid of a Zeiss Videoplan morphometric tablet (Nicol and Meinertzhagen, 1982b). The size of the presynaptic ribbon was calculated from the mean number of sections (m) in which each member of the population was visible. The area was assumed to be a rectangle with a width of (m − 1 SD) and a length of (m + 1 SD); section thickness was taken to be 70 nm (see "Results").

Results

Composition of the adult synapse. The following is a brief summary of the known anatomical features of the

adult synapse most relevant to our analysis of synaptic development. The presynaptic cellular participant is one of the six receptor terminals, R1-R6. Each terminal has many synapses all of which have a similar composition. Together these terminals form a hollow cylinder defining the extent of a single cartridge and, thereby, the geometrical boundaries of a synaptic population. The postsynaptic elements at each site comprise four members drawn in different combinations, from among five of the cells found in every cartridge. Three of these are monopolar output interneurons (L1 to L3), each of which is unique and sends an axon to the second optic neuropil. L1 and L2 contact the receptor terminals throughout the lamina (Trujillo-Cenóz, 1965; Boschek, 1971), whereas L3 does so in the distal cartridge only, i.e., furthest from the brain (Strausfeld and Campos-Ortega, 1973). The remaining postsynaptic cells are either glial (three cells surrounding each cartridge; Boschek, 1971) or α processes of anaxonic amacrine neurons (Campos-Ortega and Strausfeld, 1973; Strausfeld and Nässel, 1980) (Fig. 1*a*).

The receptor terminals have cylindrical surfaces embraced at regular intervals by the spines of L1, L2, and, distally, L3 (Fig. 1, *a* and *b*). Amacrine (α) processes run in the outer longitudinal gutters between neighboring receptor terminals and send spines into the cartridge interior, while a sheet-like glial shroud invests all cellular elements. All receptor terminals R1-R6 are equivalent, forming synapses with the same frequency and postsynaptic complement (Nicol and Meinertzhagen, 1982a).

Each synapse has a tetrad of postsynaptic processes with a bilaterally symmetrical geometry which reflects the elongate shape of the overlying presynaptic ribbon (Fig. 1*c*). Two elongate central processes, invariably L1 and L2 (Trujillo-Cenóz, 1965; Boschek, 1971), have two polar elements inserted between them at either end. The two polar processes are most often both from the same α, amacrine cell process (Fröhlich and Meinertzhagen, 1982a) and this is the combination encountered most frequently in the results of this study. In the distal cartridge only, both may be glial or may derive from an L3 in combination with either an α or a glial process (Burkhardt and Braitenberg, 1976; Nicol and Meinertzhagen, 1982a).

All of our micrograph series, with the exception of one (at 81% of pupal development), were taken too proximally in the lamina to encompass the contributions of

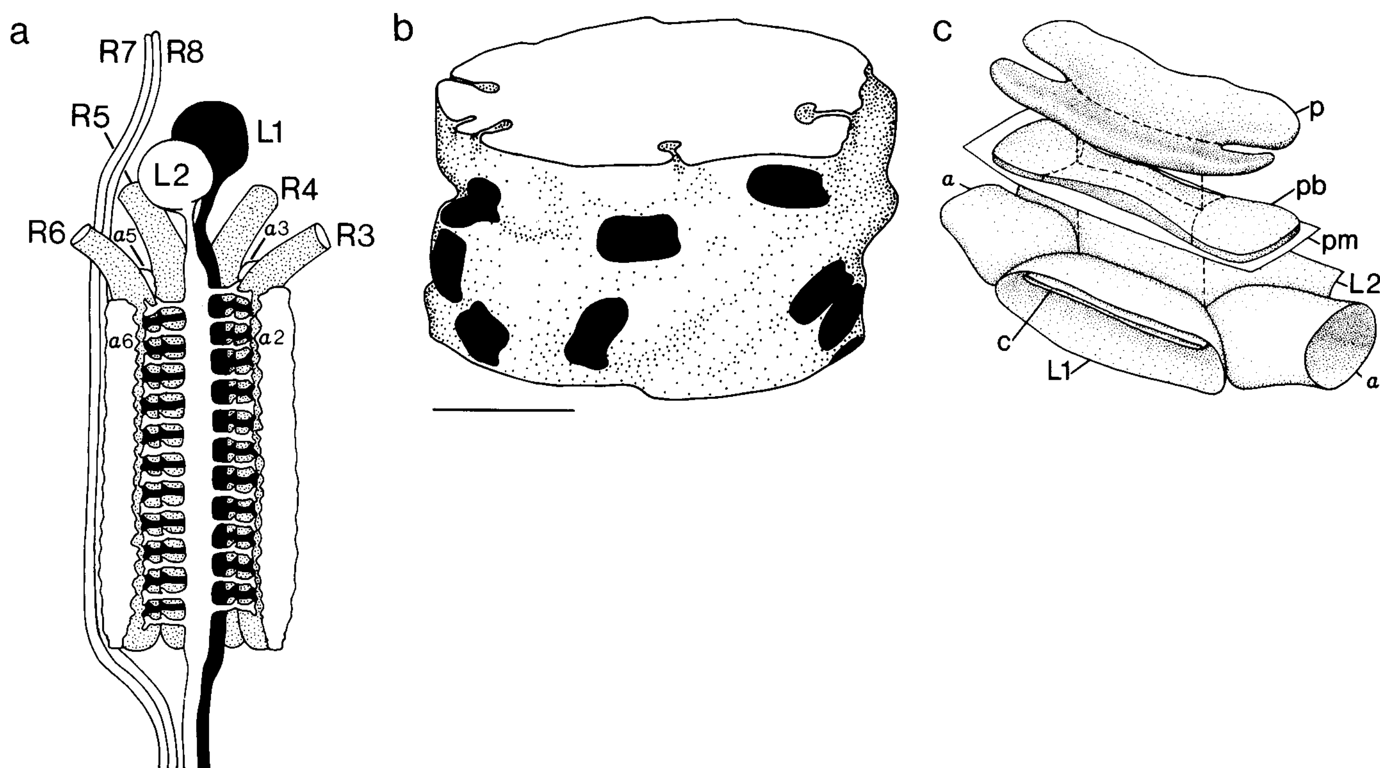

Figure 1. The cellular participants at a fly photoreceptor tetrad synapse. *a*, Schematic cutaway diagram of a cartridge in the fly's lamina showing R3 to R6, four of the six photoreceptor terminals, and L1 and L2, two of the monopolar cells of each cartridge, together with some of the α processes of amacrine cells ($\alpha 2$, $\alpha 3$, $\alpha 5$, and $\alpha 6$) postsynaptic at tetrads. The receptor axons R7 and R8 bypass the lamina without synaptic involvement (Boschek, 1971). Monopolar cell L3 and glial cells which may participate postsynaptically at distal photoreceptor tetrad synapses, as well as all other neuronal components of the cartridge not involved at these synapses, are omitted for the sake of clarity. *b*, A segment of receptor terminal R5 of a 3-week-old adult female, drawn from a computer reconstruction of 53 consecutive sections, with the presynaptic sites of receptor synapses shown in *black*. The terminal is shown from the vantage point of L1; synapses are situated preferentially on the leading edge as shown (facing the cartridge interior) and on either side (adjacent to neighboring receptor terminals). Invaginations in the *topmost* section of the reconstruction are glial-derived capitate projections (Trujillo-Cenóz, 1965). Magnification × 22,800; *calibration*, 1 μm. *c*, Exploded view of the components of a single adult synapse. A presynaptic ribbon, consisting of a platform (*p*) sitting atop a bar (*pb*), lies in close apposition to the presynaptic membrane (*pm*) of the receptor terminal. The four postsynaptic elements are *L1, L2*, α, α, of which L1 and L2 both have a postsynaptic cisterna (*c*).

L3 which ramifies distally. With the same exception they also failed to document any significant postsynaptic contribution of glial cells. This glial contribution was recorded previously, but at a depth in the lamina more distal than in the analyses presented here (depth level 2: Nicol and Meinertzhagen, 1982a). These findings thus suggest that postsynaptic glial involvement is, like that of L3, restricted to distal regions of the cartridge. We are therefore able to express the developmental transformation of the postsynaptic composition of these synapses principally with reference to a conservative tetradic complement of L1, L2, α, α.

The analysis of synaptic populations. In order to discover how each synapse comes to incorporate these four elements so precisely, populations of synapses were analyzed at developmental stages of increasing age, for both the number and composition of their postsynaptic processes. Each synapse was subsequently assigned to one of three nested categories. All synapses of the population fall into the first category, the criterion for which was simply their positive identification as a synapse from the presence of a presynaptic ribbon and synaptic vesicles. This population (N in Table I) includes a subpopulation constituting the second category, defined as that for which the number of postsynaptic elements could be scored reliably. The third category contained that subpopulation of the second at which all of the postsynaptic elements could be identified, either without doubt or with very high probability. We detected no suggestion of a bias that might have precluded our observing one or the other class of neuron at a postsynaptic location. This analysis relied, however, upon the tedious and sometimes uncertain tracing of small dendritic profiles through consecutive micrographs. Consequently, category 3 above was smaller than category 2, which was smaller than category 1. For example, most obliquely sectioned synapses are easy to identify but hard to score for the number of their postsynaptic elements. In addition, the small size of synapses in young stages caused relatively fewer to be scored in category 2, as compared with their counterparts in adult stages. Thus, the number of synapses at which all postsynaptic elements could be enumerated increased, as a proportion of the whole, from only 0.32 at 74% pupal development to 0.84 at 100% (Fröhlich and Meinertzhagen, 1982a). Despite these uncertainties, young populations were found to have, on average, fewer postsynaptic elements than did their mature, exclusively tetradic counterparts, immature monads, dyads, and triads coexisting with tetrads as late as 81% pupal development (Fig. 2).

The sequential assembly of elements at individual synapses. A simple hypothesis which accounts for the varied composition of synaptic populations in Figure 2 is that individual synapses assemble sequentially, element by element (Fröhlich and Meinertzhagen, 1982a). The following findings support this hypothesis. First, the characteristic tetradic complement of the adult synapse was never exceeded in younger stages; there were, for example, no pentads routinely formed. Second, analysis of those synapses at which the total number of elements could not be reliably counted indicated a composition qualitatively similar to that of synapses at which the total could be reliably counted. At 74% pupal development, for instance, the 68% majority of synapses which fell outside category 2 and were thus not represented in Figure 2 consisted of 25% with at least three postsynaptic elements, 33% with at least two, and 9% with at least one. Third, a preliminary analysis was undertaken of a cartridge earlier on in development, from an animal at the 62% stage of pupal development. Of 18 synapses examined in a total population of about 100, no tetrads were seen at all, no triads for certain, but 15 dyads and three monads were seen. Up to three of the probable dyads could have been triads and up to eight probable dyads could have been monads. Uncertainty existed, because of the slender ultrastructural criteria for the existence of a synapse at such a young stage, for which reason further analysis was discontinued.

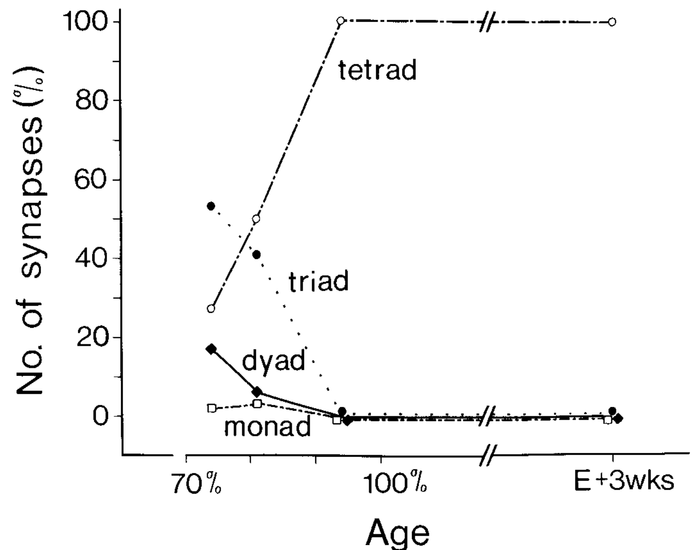

Figure 2. Proportions of synapses with one (*monad*), two (*dyad*), three (*triad*), or four (*tetrad*) postsynaptic elements, at different developmental stages (expressed as a percentage of pupal development from pupation (0%) to eclosion (E) at 100%). Synapses of a given numerical composition are expressed as percentages of the eligible category 2 population, the size of which for each developmental stage is as follows: 88 (74%), 34 (81%), 59 (94%), and 72 (3-week adult).

Given that a typical synapse were to assemble element by element, the important question becomes whether assembly proceeds in a random or in a defined sequence, since these alternatives pose different requirements of cell recognition and of the timetable of assembly. An answer to this question came from analysis of the composition of dyads and triads seen at immature stages and presumed antecedents to tetrads. These dyads and triads were not of fixed composition, as would be expected if a synapse were to assemble in a defined sequence, but had subclasses with different combinations of postsynaptic elements (Fig. 3). None of the subclasses incorporated an incorrect postsynaptic element, one that would not be represented in the adult postsynaptic tetrad. They were clearly, therefore, not chance groupings at any stage and appeared to follow a definite assembly sequence which is presented later, under "Discussion." The analysis is necessarily based upon a restricted number of synapses, those at which not only could all postsynaptic

elements be enumerated but also each element traced back to its identifying parent fiber. The synaptic populations of Figure 3 are thus in the third category, subpopulations of those in Figure 2. They represent, in the extreme case of the 74% pupal development stage, only 14% of the population of 283 synapses. In order to assess the validity of these restricted samples, the frequency with which any particular element was incorporated postsynaptically at each of the four ages analyzed is shown in Table I. This analysis disregards for the moment the number of elements contributing to a synapse and considers all synapses to which an identified postsynaptic element of any sort could be traced. The relative frequencies of contribution of L1, L2, and α elements at these expanded numbers of synapses were similar to the composition of the completely identified synapses in Figure 3. The probability of successfully tracing any one postsynaptic participant to a particular synapse increased from less than 20% to more than 80% (Table I) as synapses enlarged and differentiated between 74% pupal development and adult stages.

Three features of the combinations of elements found at these different subclasses of synapses (Fig. 3) stand out and will be discussed later. First, all dyads had at least one process of a monopolar cell, either L1 or L2, as a postsynaptic element. This general rule is contravened at only one synapse recorded, a double-α dyad found at the 74% pupal development stage (Fig. 3). We are sure of all aspects of its identification but think it an exception. Second, the single monad identified in the animal at 74% pupal development had an L1 fiber process as the postsynaptic element (Fig. 3). We are sure of this identification and have found several other putative monads, not only in the 74% but also in the 62% pupal development stage previously mentioned, but in each case we are uncertain of some aspect of their identification. Third, synapses (monads, dyads, or triads) with only a single monopolar cell process present showed no preference and incorporated either L1 or L2.

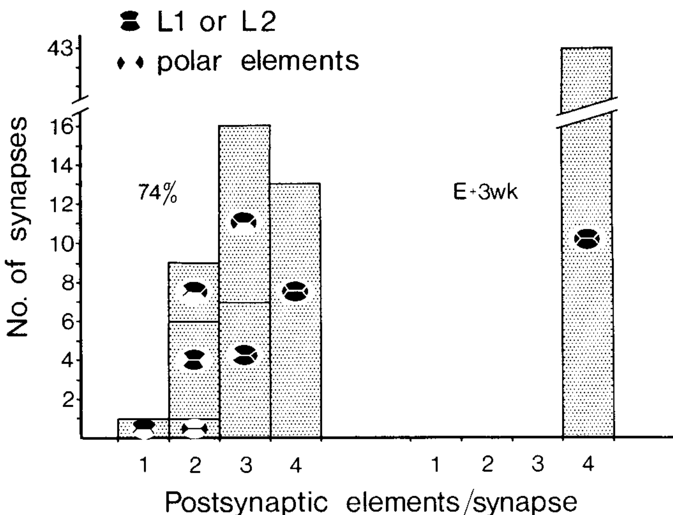

Figure 3. The number and postsynaptic composition of synapses from immature (74% pupal development) and 3-week adult females. Only those restricted populations of synapses at which both the number and the identity of postsynaptic elements were reliably ascertained are included.

TABLE I

Frequencies of synapses incorporating particular postsynaptic elements within the corresponding populations from flies of each of four ages

Frequencies are taken from counts of individual synapses (identified by the presence of a presynaptic ribbon and synaptic vesicles) expressed either as absolute numbers (n) or as their relative frequencies ($r.f.$) given as a percentage of the total identified synapse population (N). The postsynaptic elements are segregated into those elements which occupy the central positions of the mature tetrad (i.e., L1, L2, and their overlapping subset L1 + L2) and those which occupy the polar positions, i.e., α or glia (synapses containing at least one of these elements and their respective subsets containing two such elements) or L3.

Element		Age[a]							
		74%		81%		94%		E + 3 wk	
		n	*r.f.*	*n*	*r.f.*	*n*	*r.f.*	*n*	*r.f.*
L1	†[b]	35	18%	21	42%	53	80%	40	81%
	‡	15		19		19		30	
L2	†	34	18%	15	40%	58	74%	55	87%
	‡	17		23		9		20	
L1 + L2	†	19	13%	5	18%	37	58%	14	57%
	‡	18		12		15		35	
>1α	†	46	20%	5	18%	60	90%	72	87%
	‡	10		12		21		3	
2α	†	22	10%	1	3%	32	43%	46	63%
	‡	16		2		7		8	
>1 glia	†			6	29%				
	‡			22					
2 glia	†			1	8%				
	‡			7					
L3	†								
	‡								
N		283		95		90		86	
Depth[c]		2–3		2–3		3–4		3–4	

[a] Ages are expressed either as a percentage of pupal development (eclosion being 100%) or as adult post-eclosion age.

[b] †, Those synapses scored as containing the particular postsynaptic element, or elements, with certainty (as compared with an additional group, ‡, for which this identification was more doubtful).

[c] Depth levels 1 (distal) to 5 (proximal) are defined in Nicol and Meinertzhagen (1982a).

The formation of a population of synapses. We assume from the above results that synapse formation involves assembly by sequential addition of postsynaptic elements, so that monads transform to dyads, and so on. However, to be able to claim that the populations of synapses seen at each age could plausibly represent the antecedents of those seen in a different animal at the next oldest age requires at least two things: first that we neglect for the moment the earliest stages of synaptogenesis when most synapses arise *de novo*, and second that there are sufficient numbers of synapses at each stage to give rise to those seen at the next. It is therefore interesting to calculate and compare the total number of synapses present at each stage.

Extrapolation of the total population of synapses per receptor from counts in reconstructed segments of cartridge relies heavily on assumptions about section thickness, the total depth of the lamina, and homogeneity in the distribution of synapses. Mean section thickness has been taken throughout as 70 nm for each of the series analyzed in most detail (Fröhlich and Meinertzhagen, 1982a). This value was derived from an internal calibra-

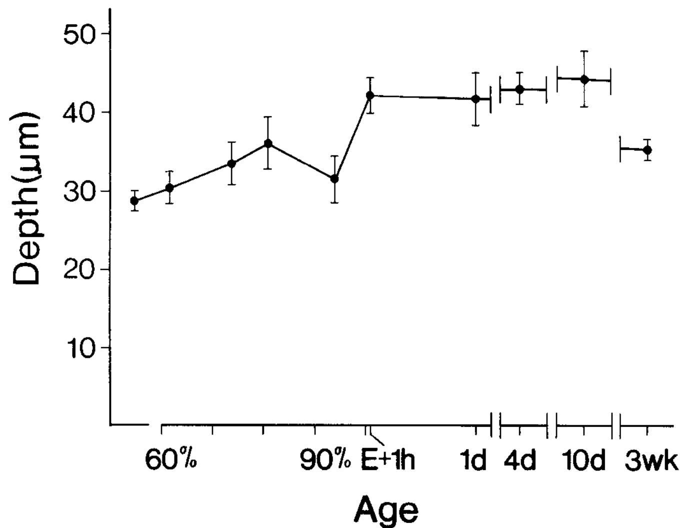

Figure 4. The depth of the anterior third of the lamina at different developmental stages. Measurements were taken only from the region of lamina sampled for synaptic analyses and correspond to the length of receptor terminals over which synapses are distributed. Mean ± SD.

tion, the standard length of the adult presynaptic bar (see Nicol and Meinertzhagen, 1982a). The overall depth of the lamina increases only gradually with age, about 15% from 74% pupal development to adult stages (Fig. 4). The wide scatter in these values probably equals but does not exceed other sources of variance in calculating the number of synapses, notably from the calculation of section thickness and the contribution from inter-receptor variability. We also have no systematic evidence of the distribution of synapses down the length of the immature receptor terminals to know quantitatively how reliable an estimate based on a sample made at one depth level only might be. In the adult, there is depth-dependent variation in the frequency of synapses along the receptor terminal, middle depths having around 20% fewer synapses than distal or proximal regions (Nicol and Meinertzhagen, 1982a). Any variation that might also exist in younger stages is unlikely to be of greater magnitude, since longitudinal sections of cartridges at 81% pupal development revealed interneuronal dendrites evenly distributed along the length of the terminal. In particular, no bald spots or other local inhomogeneities in synapse frequency were found, the presence of which might grossly confound our estimates. These were in any case based on samples all at approximately the same depth level in the cartridge (Table I), as defined by the criteria of Nicol and Meinertzhagen (1982a).

The estimates of the sizes of synapse populations show that, after an initial increase, starting at 74% pupal development there were actually more synapses per receptor for each developmental stage than were found in the next older stage (Fig. 5). In other words, during the last quarter of pupal development some synapses must be lost from each receptor. The fact that formation of new synapses probably continues after the reduction in population size has commenced (we identified a monad still at 81% of pupal development, for example) suggests, however, that the loss should properly be considered a net loss. The net loss of synapses ceased around eclosion, the number increasing to a second maximum (Fig. 5) at around 1 day, thereafter declining again to reach a value in a 3-week adult about the same as at eclosion. These post-eclosion changes, however, probably fall within the limits of accuracy of our determination of the size of a synapse population and thus may not be real.

Temporal aspects of synapse formation. More exact information on the timetable of synaptogenesis comes from counts of synapses with a given numerical composition (dyad, triad, etc.) at each of the ages studied (Fig. 6, Table II). Even if we choose to ignore variability in

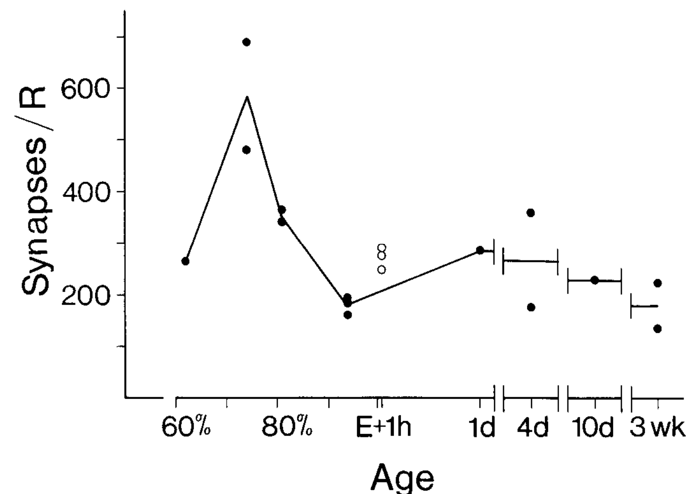

Figure 5. Mean synapse population size for receptor terminals of different ages. Each value (*solid circles*) is derived from counts of part of a serially sectioned cartridge, projected upon the entire length of the terminal. *Open circles* are data calculated by us from serial section analyses of cartridges S4C1, S4C2, and S2C1 of which the first value was reported in Nicol and Meinertzhagen (Table 5, 1982a).

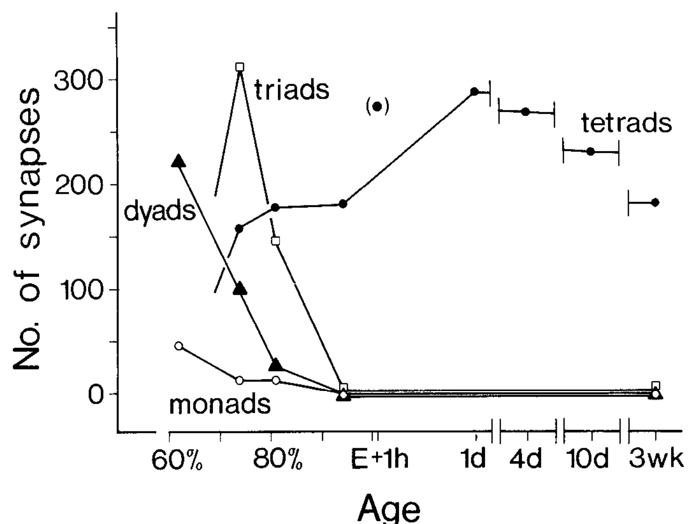

Figure 6. The number of synapses with one (○), two (▲), three (□), or four (●), postsynaptic elements in receptor terminals of different ages. Values are derived from those shown in Figures 2 and 5. For *tetrad* synapses the value enclosed in *brackets* is the mean of the three values of Nicol and Meinertzhagen (1982a) shown by open circles in Figure 5.

TABLE II

Absolute numbers of synapse and membrane area and the differences between corresponding values in animals of consecutive age groups

Age		Number of Synapses					Membrane Area (μm^2)
Hours	Stage	per Receptor	Monad	Dyad	Triad	Tetrad	
73	62%	265					164
(14)[a]		(321)					(206)
87	74%	586	12	99	313	159	370
(8)		(−234)		(−77)	(−168)	(15)	(−42)
95	81%	352	11	22	145	174	328
(15)		(−173)					(−71)
110	94%	179	0	0	0	179	257
							(127)
E + 3wk	100%+	180	0	0	0	180	384

[a] Values in parentheses represent differences between corresponding values in animals of consecutive age groups.

these populations (i.e., variability between animals, between cartridges, between depth levels in the lamina) in order to make comparisons at different times, the problem remains to know what fraction of synapses at an older age actually survived from a younger one. Synapses may form *de novo* in the time period between the two samples and so be scored only in the older; alternatively, they may be lost between samples and so be scored only in the younger. If present in both populations they may either persist unchanged or have acquired one or more additional postsynaptic elements and so be scored in a different category. Despite this fluidity, some conclusions are unambiguous. Individual synapses can apparently form quickly. There were, for example, more triad synapses at 74% pupal development than there were synapses of all classes at 62 % pupal development, only 14 hr earlier (Table II). Thus some triads must have formed wholly within this period, adding their postsynaptic elements at least as fast, on average, as one per 7 hr or so. Similarly, since only monads and dyads were found at 62% pupal development, the tetrads at 74% pupal development must have formed at least from the dyads existing at the earlier stage. These tetrads (about 160 in all, or 27% of the population) must also, therefore, have incorporated their two additional postsynaptic elements at an average rate of at least one per 7 hr.

Synapses are formed rapidly *de novo*, too. In the transition between these same two populations at 62% and 74% pupal development an additional 320 synapses per receptor had formed (Table II). To produce this increase about 23 must have formed per hour, along with 14.7 μm^2 of new receptor terminal membrane added per hour between the two stages (Fig. 10). Similarly, synapses can disappear in large number over short periods. For example, in the 8-hr period between 74% and 81% of pupal development, a net 230 synapses approximately were lost from each receptor (Table II) (i.e., 29/hr, corresponding to the net removal of at least one synapse/13 μm^2 of original receptor terminal membrane an hour). In the 15-hr period between 81% and 94% pupal development, this loss rate was reduced to 12 synapses/terminal an hour or 1 synapse/27 μm^2 of membrane an hour, respectively. Loss of individual synapses apparently does not occur by sequential disassembly, the reverse process of their construction, because no larger numbers of monads were seen at 81% than were seen at 74% pupal development—yet in the developmental transition between the two, a net loss of about 80 dyads and 170 triads per receptor occurred (Table II) for the net gain of only 15 tetrads. Sequential disassembly could then occur only if very rapid, given that, at this time, about 40 synapses were lost per hour, as explained earlier. We have detected no ultrastructural characteristics suggestive of individual synapses undergoing either assembly or disassembly.

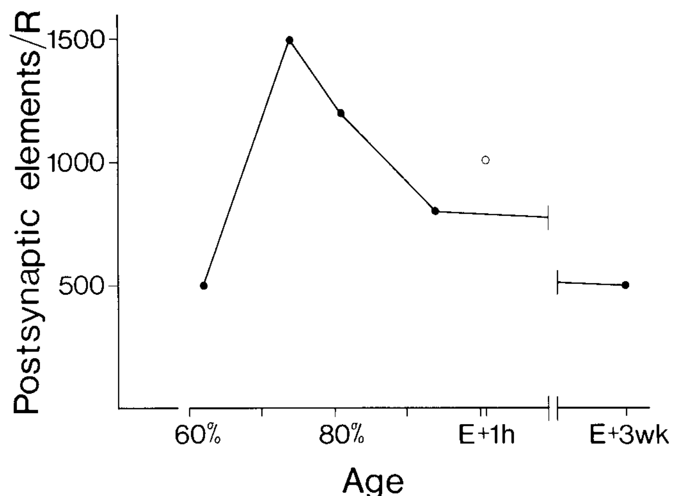

Figure 7. The mean total numbers of processes postsynaptic at all synapses of a receptor terminal at different ages. Values are derived from those shown in Figures 2 and 5 and rounded to the nearest 100. The *open circle* is calculated from the serial section analysis of cartridge S4C1 in Nicol and Meinertzhagen (1982a).

Despite the fact that the populations of receptor synapses undergo important developmental fluctuations, their constancy in the adult (Nicol and Meinertzhagen, 1982a) suggests that each developmental stage may be actively regulated. Although individual synapses increase the number of their postsynaptic elements during development, it could be that the total postsynaptic involvement at those synapses does not actually change, if this increase is compensated for by the overall decrease which occurs in the receptor's synapse population. This is apparently not the case (Fig. 7). The most postsynaptic elements were at the 74% pupal development stage and the number decreased thereafter, indicating not only that the receptor's population of synapses decreased during the last part of synaptogenesis but also that the postsynaptic cells underwent a decrease in synaptic involvement.

The decrease in both cases was, numerically speaking, considerable and was attended by an increase in the size of those synapses which persisted (Fröhlich and Meinertzhagen, 1982a).

Size of individual synapses. As synapses age, they grow, both in size and in the number of their postsynaptic elements (Fröhlich and Meinertzhagen, 1982a). A simple comparative measure of this increase is provided by the size of the presynaptic ribbon, expressed as the mean number of consecutive sections each occupied. These values are provided in Figure 8 for all synapses of the different age groups. They are somewhat higher than, on average, and perhaps overestimate the area of the ribbon as compared with those calculated from the data in Fröhlich and Meinertzhagen (1982a). Nevertheless, both sets of values showed comparable increases with age. Because the young stages contained dyads and triads as well as the tetrads found exclusively later on, it is also of interest to compare just the tetrads at each age (Fig. 8). The size of presynaptic ribbons at tetrad synapses increased during development (Figs. 8 and 9), suggesting that the growth of individual synapses proceeds not only by the accretion of postsynaptic elements (Fig. 9) but also by an increase in size of the synapse itself. In all cases, however, variability in size among the synapses of a given numerical composition, tetrads for instance, was greater than the difference in mean size between such groups, e.g., between triads and tetrads.

Since the individual synapse increases in size during development, it may be that, after the initial rise, the total area of all synaptic contacts is conserved, increase in size being gained at the cost of number. Total synapse area, measured as the product of synapse number and mean presynaptic ribbon area, was not, however, constant with age; its absolute value showed an early rise at 74% pupal development, peaking at the 1-hr adult. The cumulative area of the presynaptic ribbons measures only the effective synaptic area, however, and fails to account for the increase in area which occurs in the membrane as a whole, over which the synapses are distributed and which results from the growth of the receptor terminal. Measurements of presynaptic membrane area of the receptor terminals showed that the fraction of presynaptic membrane actually occupied by synaptic sites rises initially, peaking at about 10% of the total membrane area (Fig. 10).

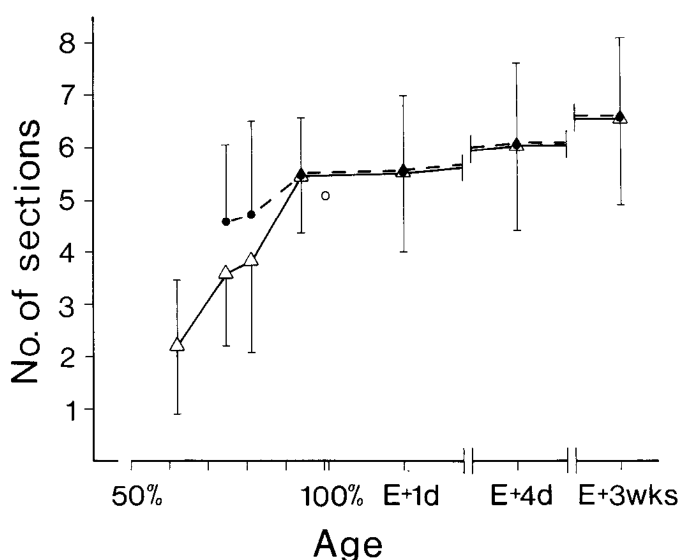

Figure 8. The mean extent of presynaptic ribbons at different developmental stages. △, mean values (in number of consecutive sections ± SD) for all synapses of a developmental stage; ●, mean values for tetrad synapses only. Section thickness is taken to be 70 nm. The *open circle* is the value reported in Nicol and Meinertzhagen (1982a).

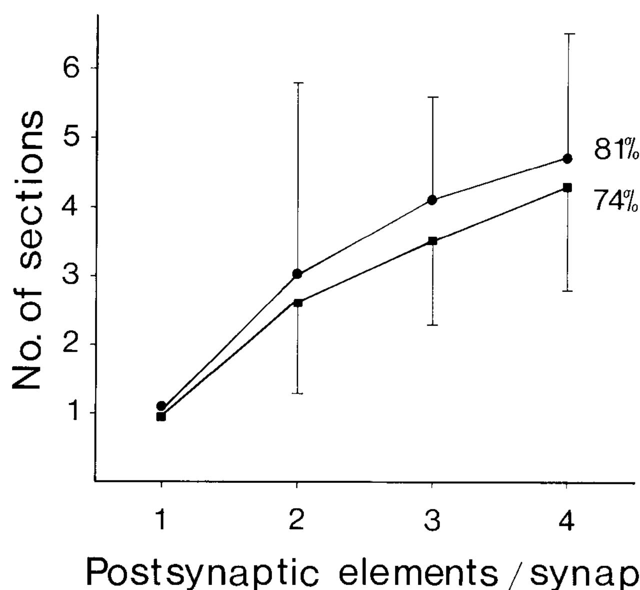

Figure 9. The mean extent of presynaptic ribbons for monads, dyads, triads, and tetrads of 74% and 81% pupal development stages. Mean ribbon size increases with the number of postsynaptic elements and also, somewhat, between the two developmental stages. Values are mean number of consecutive sections ± SD.

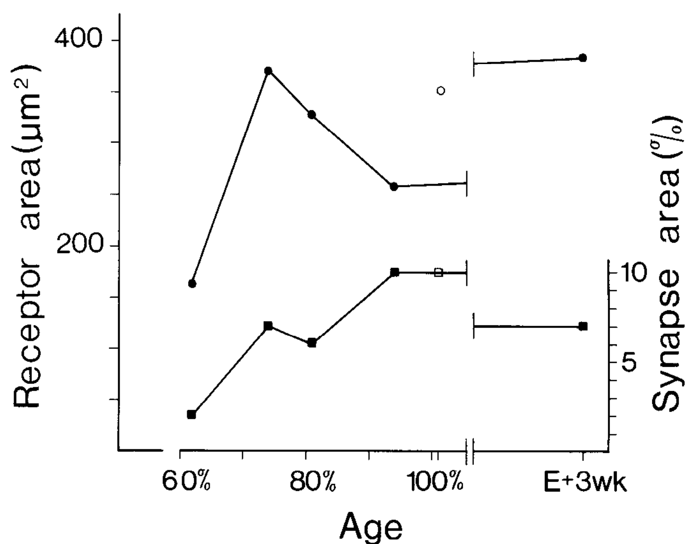

Figure 10. Upper curve, Mean membrane area of a receptor terminal at different developmental stages. *Lower curve,* Sum of all presynaptic ribbon areas, expressed as a proportion of the receptor terminal membrane area, at different developmental stages. *Open symbols* are the corresponding 1-hr adult values calculated from the serial section data of Nicol and Meinertzhagen (1982a).

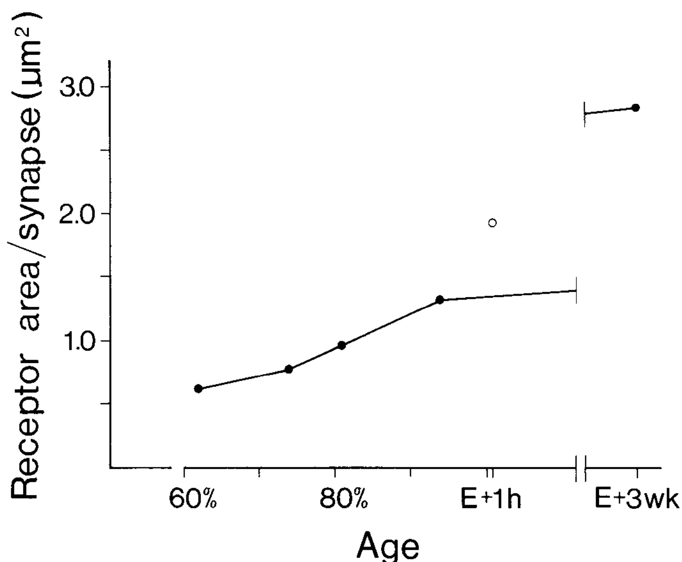

Figure 11. Mean ratio (A/N) of the membrane area of a receptor terminal to the number of synapses formed by the terminal at different developmental stages. The *open circle* is the value derived from serial section analyses of cartridge S4C1, given in Nicol and Meinertzhagen (1982a).

The size relationships between synapses and receptor terminals. A close proportionality can be demonstrated between synapse number and the associated membrane area of the adult receptor terminal (Nicol and Meinertzhagen, 1982a, b). The average ratio of membrane area to its synapse number (A/N), i.e., the area of membrane "allotted," on average, to each synapse (Fig. 11), is readily derived from the data already provided (Figs. 5 and 10, Table II). The value of A/N increased regularly with age, slowing somewhat in adult flies. From this relationship and a similar one derived earlier for the mean area of presynaptic ribbon per synapse (Fig. 8) a further, and we believe more basic, relationship emerges. If A/N values are compared to the mean ribbon areas per synapse, a simple proportionality is apparent for each age examined (Fig. 12). Thus the area of a synapse itself is proportional to the area of membrane allocated to it, on average, at all stages of its growth pre- and post-eclosion. This should not, however, be construed in any way to describe the spacing of individual synapses, which we have not yet studied definitively.

A similar age dependence can also be demonstrated in the relation of the receptor terminal volume to synapse number (Fig. 13). The similarity of the area and volume relations results from the concomitant changes in receptor terminal membrane area and volume occurring during development. The expected nonlinearity of changes in volume, as compared with surface area, does not appear in the data points available. The nonlinearity is, in any case, modified by changes in shape of the cells (older terminals have smoother, more regular morphologies than do their immature forms). Perhaps for such relatively small changes in receptor terminal diameter as occur during the development stages we have examined, nonlinearity is undetectable within the scatter of the points. As a consequence of this indeterminacy, therefore, there is also a simple proportionality between the area of the presynaptic ribbon and the mean volume of receptor terminal per synapse (Fig. 14).

Various factors could affect the exact values derived for synapse area and volume functions. Differences in fixation and dehydration treatments of different tissue samples, although performed according to a standard schedule, could presumably produce volume changes that would also affect the individual A/N ratios, as could the differential sensitivity of tissues of different age to these treatments. The likelihood of subsequently deriving a linear relationship from the original measurements in the presence of a confounding influence, such as variable osmotic swelling or shrinkage, would, however, seem remote. For this reason we think the linear relationship is authentic.

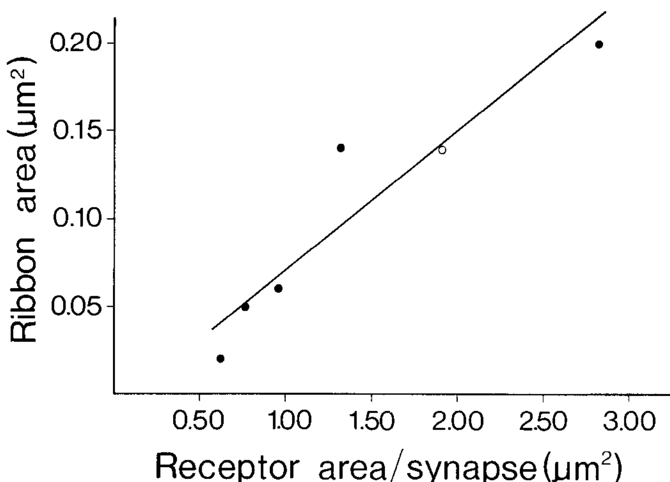

Figure 12. Mean area of the presynaptic ribbon as a function of the mean area of receptor terminal membrane "allotted" to each synapse (the ratio A/N in Fig. 11) for different developmental and adult stages. Correlation coefficient = 0.942, $0.01 > p > 0.001$. The *open circle* is the value calculated from the analysis of Nicol and Meinertzhagen (1982a). Note that data points form a chronological sequence along the *regression line* and that the latter cannot hold for values near the origin. Data in this figure have been incorporated in a previous report (Fig. 6 in Meinertzhagen and Fröhlich, 1983).

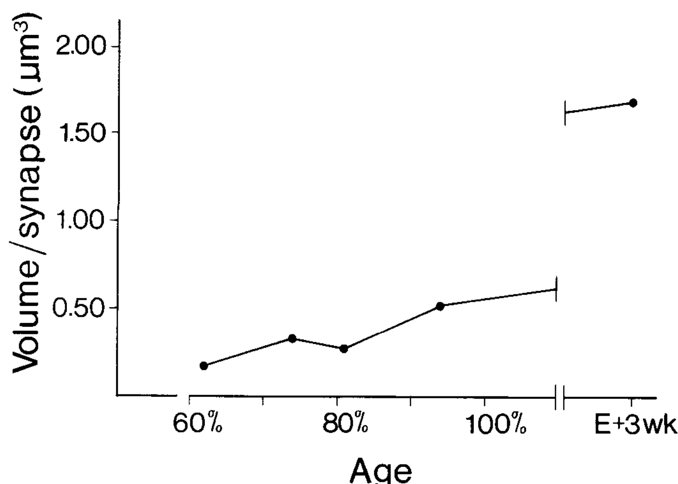

Figure 13. Mean ratio of the volume of a receptor terminal to the number of synapses formed by the terminal at different developmental stages.

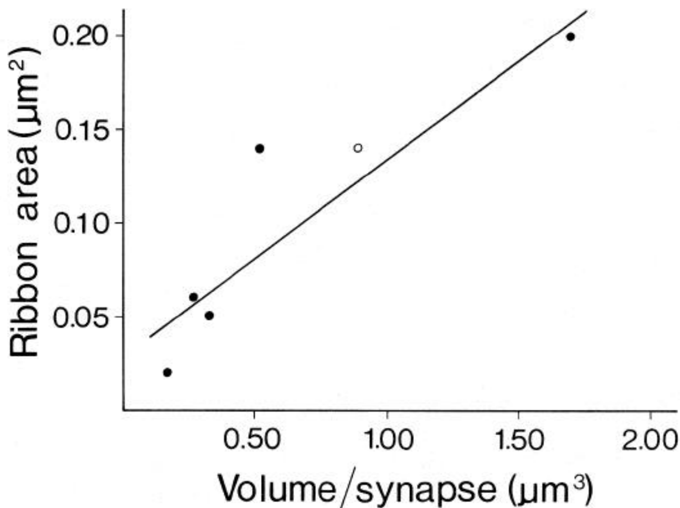

Figure 14. Mean area of the presynaptic ribbon as a function of the mean volume of receptor terminal "allotted" to each synapse (from Fig. 13) for different developmental and adult stages. Correlation coefficient = 0.903, $0.05 > p > 0.01$. The *open circle* is the value calculated from the analysis of Nicol and Meinertzhagen (1982a).

Discussion

The sequence of addition of elements at individual synapses. Immature synapse populations in the fly's lamina have a composition which suggests that their individual synapses form by the sequential addition of postsynaptic elements at the presynaptic site. Since incorrect and supernumerary combinations of elements were almost never observed, the process of addition must be highly selective; the many other cells present in the cartridge, for example, never appear in postsynaptic locations of the emerging tetrads, although they synapse nearby. The sequence of addition which could account for all observed combinations of elements in synapses is shown in Figure 15. It is based upon the completely identified synapses recorded in Figure 3 and is supported by analyses of all other synapses (summarized in Table I). The double-α dyad previously noted in Figure 3 is a sole exception at variance with this assembly pathway. Synapse formation is initiated at a R → L monad (Fig. 15). Much of the strength of this assumption rests on the classes of dyads and triads observed, in view of the very few monads identified with certainty. With the exception of the double-α dyad, all of the dyads and triads possessed one or two L processes, providing further support for interpreting the former as atypical and for assuming that the first contact to initiate a synapse occurs between the receptor terminal and an L cell. L1 and L2 are apparently equivalent in their capacity to make this initial contact, since either may be encountered at synapses which contain only a single L cell process.

Whereas many possible combinations of growing neurites could occur at synapses by chance, only two classes each of triads and of dyads are actually encountered. Both types of triad could be antecedents of the single (adult) form of tetrad by recruitment of the missing postsynaptic element, whereas both types of dyad could be antecedents of the two types of triad by a similar addition. There are thus only three actual sequences of consecutive single-element additions to a R → L monad: L, α, α; α, L, α; and α, α, L.

Selection of the exact sequence of construction of a synapse apparently rests with the local availability of the postsynaptic elements since all combinations have been found. All intended postsynaptic cells are present throughout synaptogenesis, with the exception of glial cells which invade the cartridge interior only extensively after 74% pupal development (Fröhlich and Meinertzhagen, 1982a). Within the local territory of a synapse, within, say, a 1-μm radius, there appears to be no preferred pathway by which postsynaptic elements may gain access to a presynaptic site, and we therefore assume that initial contact occurs by chance encounter. The ultimate requirement to bring together four postsynaptic elements of the correct composition, however, may somewhat favor certain final sites for synapse formation. For example, for the minority of synapses formed upon the main axon trunks of adult L1 and L2, preferred locations exist.

Although the sequence of addition of postsynaptic elements at individual synapses seems to be arrived at by chance, we infer the operation of several developmental rules to ensure that the different pathways of assembly lead to a standard tetradic outcome. These rules therefore seem to operate as exclusion principles allowing all postsynaptic combinations to occur that are not expressly excluded, so enabling high precision, eco-

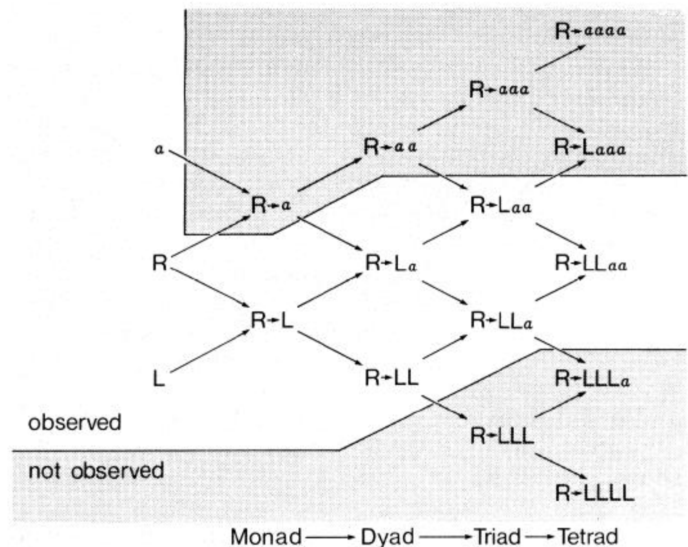

Figure 15. Scheme of the possible pathways of sequential assembly of single postsynaptic elements at synapses with one (*monad*), two (*dyad*), or three (*triad*) existing elements. Monopolar cell processes (L1 or L2) are represented only by *L*. Starting with the three cellular participants at synapses in the proximal lamina, i.e., with *R*, *L*, or α, the total number of possible pathways diverges with each single-element addition. Of these, only those synapses are observed that could have been initiated as R → L monads and could also give rise to the single class of tetrad of the adult. *Arrows* represent straightforward presumed pathways of accretion of processes, as synaptic complexity increases. We cannot exclude more complex sequences involving both deletions and extra additions, but we have no evidence of these.

nomical quality control of synapse assembly, without the need for detailed control of neurite movements or for frequent reversals in assembly sequences. Thus, in addition to general instructions concerning the timetable of postsynaptic neurite growth, the major operational instructions must involve cell recognition so as to preclude contacts from forming from most cells in the cartridge at any one instant in the life history of any one synapse.

The minimal requirements for cell recognition according to the scheme of Figure 15 are, therefore, that:

1. Recognition must be possible between R and L (either L1 or L2), to initiate synaptogenesis.

2. Recognition must be possible between α and an R-L complex.

3. An exclusion principle must exist for >1 L1 or >1 L2 process at any one synapse in order to exclude L1/L1 or L2/L2 combinations, which we never found.

4. An exclusion principle must exist for >2 α processes at any one synapse to preempt the formation of pentads.

5. In addition, there must be further rules, since in the distal lamina processes from L3 or glial cells substitute for α profiles at some synapses (Nicol and Meinertzhagen, 1982a). These rules normally prevent α and glia from cooperating at a single synapse or L3 from contributing twice at a single synapse.

The fixed spatial geometry of the tetrad cannot result from the exact sequence with which elements are added postsynaptically, since this sequence is flexible. The exact spatial arrangement of the tetrad must therefore be the product of interactions occurring between the group of elements *in situ*, the consequence of which is that α elements (or their substitutes) are never neighbors in the tetrad. One feature of these interactions is that they are independent of the exact contributors to the polar positions of the tetrad, α, glial, or L3, since all tetrads assume the same final geometric pattern. The pattern is remarkable in most cases where two α processes contribute the polar elements of the tetrad, since these normally come as double contacts from a single dendrite. It seems, therefore, that the invariance of the tetrad's geometry must result from one or another of its fixed features, the pairing of L1 with L2 and/or the presynaptic receptor site itself.

The timetable we have estimated for assembling individual synaptic elements indicates the addition of postsynaptic elements at intervals of up to 7 hr, on average. This time falls inside the period of 12 to 18 hr reported from the definitive timing studies of Rees et al. (1976) for the appearance of postsynaptic densities and synaptic vesicles following first neurite contact *in vitro*. For fly synaptogenesis *in vivo*, general contact between intended pre- and postsynaptic neurons is of course established long before synaptogenesis (Trujillo-Cenóz and Melamed, 1973; Hanson, in Kankel et al., 1980), which is apparently postponed until the brief interval of intense synapse formation, concentrated around the period represented by our analyses at 62% and 74% pupal development. The recently reported temporal pattern of synaptogenesis in the primary sensory synaptic region of another insect, the glomerular neuropil of the antennal lobe in the moth *Manduca*, has, by contrast, a phase of rapid synapse formation earlier in pupal development (Tolbert et al., 1983).

The maximal rates of formation of synapses which we have found (23 synapses/hr between 62% and 74% pupal development), although large, are matched by examples in vertebrate retina. Fisher (1976) calculated that amacrine cells form presynaptic associations at a maximum rate of 26.9 synapses/hr in premetamorphic *Xenopus*, and that bipolar cells form ribbon synapses at a rate of 13.7/hr. Although the closest available, these comparisons fail to take into account such obvious interphyletic differences as cell size, heterogeneity of synapse subclasses, and the metamorphic timetable of development. A similar comparison can be made to the average rate of formation of postsynaptic association by neurons of the cat's visual cortex, which is maximal between 19 and 27 days after birth at about 20 synapses/cortical neuron an hour (Cragg, 1972).

Synapse loss. There is a clear and substantial reduction during development in the number of photoreceptor synapses. This study is not, of course, a total analysis of receptor connectivities since other synaptic classes exist involving one or more of the receptor, L1, L2, or amacrine cells (Strausfeld and Campos-Ortega, 1977). In view of the small relative numbers of these (Hauser-Holschuh, 1975), however, it seems unlikely that their pattern of emergence could offset any developmental transformations we have seen in the tetrad population size.

Synapse loss may be of widespread occurrence in the development of other neural systems, but, in general, none has been analyzed with the same precision as is possible for fly photoreceptors. Studies of regional synaptogenesis in vertebrate brain, for example, often incorporate small post-peak declines in synapse frequency (e.g., Aghajanian and Bloom, 1967) but are not interpretable as changes in the populations of individual classes of synapse upon particular identified neurons. Studies on the visual cortex of mammals (Cragg, 1972) demonstrate, more specifically, a late reduction in the average number of synapses at which the average neuron is postsynaptic. All such measures of developing synaptic populations incorporate a heterogeneity of synaptic classes as well as unquantified changes in cell morphology. More direct comparison with our data is available in one formally equivalent study, on the developing receptors of the retina in *Xenopus* (Witkovsky and Powell, 1981). In this case, however, no compelling evidence was found for a developmental decrease in the population size of a receptor's synapses, with the possible exception of cone basal synapses.

The loss of synapses described here is to be distinguished carefully from the synapse elimination encountered in a number of systems, notably the neuromuscular junction (Purves and Lichtman, 1980; Stephens and Govind, 1981; Van Essen, 1982). In the fly, *synapse loss* is actually a reduction in the potential number of unitary sites of transmission at what is, in effect, a single distributed synapse established repetitively between the same set of cells. *Synapse elimination*, on the other hand, refers to the progressive developmental restriction of the number of presynaptic axons innervating a particular cell, without specific reference to the number of synapses

established between each. Some form of equivalent restriction also occurs during growth of the fly's receptor axons but earlier than in the analyses presented here. During the first half of pupal development, the growth cone of each receptor axon establishes filopodial contacts with the growth cones of a number of other receptor axons at the distal face of the lamina (Hanson, in Kankel et al., 1980) and thus may represent the receptor axon in several future cartridges. The retraction of all but one of these growth cone contacts in the fly's lamina resembles, but differs definitively from, synapse elimination at the neuromuscular junction. The crucial difference is that synaptogenesis in the fly is deferred until all intended synaptic partners are correctly juxtaposed while, in the neuromuscular junction, the phases of large-scale axonal growth and regression overlap the timetable for the formation of functional synaptic connections. There are two further differences between the two systems. First, for their respective populations of synapses, the arrangement of presynaptic sites in the fly photoreceptor is being compared with that of the postsynaptic sites on vertebrate muscle fibers. Second, in the fly the change in synapse number is not dependent upon shifts in innervation between cells but on shifts between sites on the surface of the same cell.

The finding of synapse loss, and especially the temporal overlap between synapse formation and synapse loss, forces consideration of the question of synapse turnover. Two forms of synapse turnover may be involved in the population: first, the formation and subsequent loss of synapses which fail to collect all elements of their tetrad and thus have a transitory existence only during the early stages of synaptogensis (before 94% pupal development, when dyads and triads are present); second, the formation and loss of synapses which have already become complete tetrads and may therefore have had a relatively long life after 62% pupal development, when tetrads first start to appear. The whole question of synapse turnover and net synapse number need not necessarily apply to the population of tetrad synapses, since these show no overall decrease during development before eclosion and no confirmed post-eclosion decrease (Fig. 6, Table II). Consequently, once formed, each tetrad could quite possibly persist thereafter. Direct ultrastructural evidence for these different phases of synapse formation or loss is, however, totally lacking in the fly, unlike the ultrastructural evidence reported by Wernig et al. (1980) and Bixby (1981) and interpreted as the disestablishment of synaptic contacts in mature vertebrate muscle. The breakdown of synapses in the fly is cataclasmic, either with no sequential disassembly of the postsynaptic elements or with a very rapid one.

The significance of such a large developmental loss of potential synaptic sites over the receptor terminal provides interesting grounds for speculation. The loss is not related to securing an input from neurons which are functionally correct, since synapses form only between the correct cell partners. It is possibly connected with establishing the correct strength of connection between such partners; functionally, the loss cuts back the population of mature synapses and thus adjusts the range of presynaptic signals over which a receptor terminal transmits to its postsynaptic neurons (Shaw, 1981). The outcome of the loss is that fewer final synaptic sites are selected from a larger number of possible ones. Some presynaptic sites may be geometrically favored by having a higher probability of collecting the appropriate complement of postsynaptic elements within the imposed deadline and thus are more likely to persist into the adult. Thus, it may be more appropriate to view synapse loss as an initial overproduction of synapses, a strategy to ensure the establishment of sufficient correctly constituted synapses in a given time, given the rigidity of the rules of constructing synapses.

Control of synapse number. Our results reveal the direct proportionality between the mean area of the presynaptic ribbon and the ratio of a receptor terminal's size (either its surface area or volume) to its synapse number (Figs. 12 and 14). Age-dependent changes in these quantities therefore, in effect, reveal the relationship between receptor terminal size (surface area or volume) and the cumulative area of its synapse population (the product of synapse number and the mean ribbon area). These linear relationships cannot, of course, be true for the initial phase of synaptogenesis, when most synapses form *de novo*. In the extreme case, before any synapse emerges (ribbon area = 0), the receptor area per synapse (Fig. 12) would, for example, tend toward infinity. In other words, the relationships during the initial phase of synaptogenesis must be described by a curve with a negative slope. We presume this transforms into the positive slope of the later, regulative relationship of maturing synapses around 62% pupal development. The latter is the only stage examined during the initial growth in synaptic population size (Fig. 5) and it yields the data point nearest the origin in Figure 12.

The linear relationship of Figure 12 implies that a constant fraction of the total available presynaptic membrane is devoted to synapses. This is not clearly borne out by the results plotted in Figure 10, where synapse area expressed as a proportion of membrane area falls between 6 and 10%, with the exception of a much lower value (2.4%) for 62% pupal development. However, we think that the values for older synapses fall within the range of constancy for our measurement errors. On the other hand, we attribute the increase between 62% and 74% pupal development to the large number of synapses arising *de novo*.

A previous study has shown the proportionality between membrane area (A) and synapse number (N) for adult receptor axons of a single age (Nicol and Meinertzhagen, 1982a). The present study now shows that the particular A/N quotient is age dependent and that its correlate is synapse size; as the average synapse gets older it gets larger and occupies a larger fraction of the presynaptic membrane. Studies in several other systems now also reveal a proportionality between the surface area of a neuron and the summed area of the synaptic contacts upon it. Adult parasympathetic neurons of the *Xenopus*' cardiac ganglion, for example, receive presynaptic boutons in a number proportional to, and so as to cover a constant fraction (2%) of, the surface area of their somata (Sargent, 1983). Similarly, developing locust muscle fibers reveal A/N quotients of similar mag-

nitude in the nymph and adult despite wide differences in A and N (Walther, 1981). Both examples suggest the regulation of A/N quotients of postsynaptic cells, a conclusion previously reached for the spines of L1 and L2 at fly tetrad synapses (Nicol and Meinertzhagen, 1982b). These findings imply that it is the receptor terminal's area which may be the quantity regulated during synaptic maturation; its volume may be proportional to area only fortuitously, for small size and shape changes of a simple cylinder. We therefore suggest that the correlation between mean presynaptic ribbon area and the quotient A/N is the significant outcome, and a likely determinant, of synaptogenesis. This relationship certainly describes the size and overall spacing of synapses at later stages of development and adulthood. Therefore, it seems that it is not the number of synapses per se that is the set-point of regulation but the ratio between total synaptic area and total membrane area of the receptor. Our measurements of these quantities, taken together with those of Nicol and Meinertzhagen (1982b), suggest that, for any particular age, the receptor terminal may vary both synapse number and surface area to achieve the desired ratio of A/N, whereas synapse size may be solely age dependent. The correlation established between A and N involves the mean values of both quantities, which suggests that both are regulated for the population as a whole. We make no claim about the regularity of synapse spacing since we are currently unable to plot the correlation for size and territory of individual synapses. In preliminary evidence from freeze-fracture studies, synapses appear not to be evenly spaced as individuals. This seems to rule out regulation of synapse number solely by the inhibitory effect of one synapse upon synaptogenesis in its vicinity, a conclusion also reached for *Xenopus*' cardiac ganglion cells (Sargent, 1983).

Factors influencing the absolute magnitude of A/N are still unknown, although preliminary evidence now suggests that the extent of synaptic activity may be one determinant. Receptor terminals in *Calliphora* are known to be capable of transmitting to postsynaptic neurons at times corresponding to stages examined here (Järvilehto and Finell, 1983). Furthermore, functional plasticity has been demonstrated in interneurons in the developing locust's visual system (Bloom and Atwood, 1980). In the bee, the number of synaptic contacts established by the terminals of green-sensitive receptors is reduced in animals previously reared in ultraviolet light (Hertel, 1983). This reduction is accompanied by an increase in the mean spacing distance between synapses and thus modifies the quotient A/N. We are currently examining *Musca* for similar influences of light and dark rearing.

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