Neuron Duplications and Deletions in Locust Clones and Clutches

Abstract. *Duplications and deletions of identified neurons can occur with a high degree of genetic control and specificity, as shown by examining the ocellar interneurons of locusts from different breeding populations, clutches from single mated pairs, and isogenic animals from parthenogenetic clones.*

Just as "no one supposes that all individuals of the same species are cast in the same mould" (*1*), so no one ought to expect phenotypic uniformity in the nervous systems of animals whose sexual reproduction assures genotypic variability. Although identified neurons are defined as constant and unique from animal to animal within the same species, we might expect to find phenotypic variability in the number, morphology, and physiology of identified neurons if we examined them in enough sexually reproduced animals of the same species. Investigators have recently reported two forms of variability in the number of identified neurons: (i) duplications, for example, in the sensory and motoneurons of the leech (*2*), in the large and small ocellar interneurons of the locust (*3*), and of R2 and other giant neurons in *Aplysia* (*4*); and (ii) deletions in the giant horizontal and vertical interneurons in the fly eye (*5*). The paucity of reports, particularly of deletions, may simply reflect the inherent difficulty in demonstrating these phenomena unequivocally.

In this report I describe the detection, specificity, and genetic basis of duplications and deletions of identified ocellar interneurons in the brain of the locust. I examined these cells in three types of animals: (i) animals from two different breeding populations and their offspring, (ii) the offspring within clutches from single mated pairs of known phenotypes, and (iii) isogenic animals from the parthenogenetic clones of single unmated females.

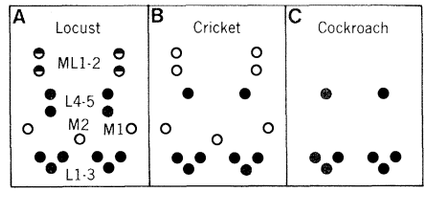

Fig. 1. Schematic diagram of the number and position of somata of the large ocellar interneurons in the brain of (A) the locust, *Schistocerca nitens*; (B) the cricket, *Gryllus bimaculatus*; and (C) the cockroach, *Leucophaea maderae*. The locust has three ocelli (two lateral and one median) and three ocellar nerves, each containing the axons of seven large interneurons. Symbols: ●, cells with axons in the left or right lateral ocellar nerve; ○, cells with axons in the median ocellar nerve; ◓, cells with axons in both a lateral and the median ocellar nerve. The cricket has only one soma where the locust has a two-cell cluster, L4–5; the axons of ML1–2, though extending through the median ocellar nerve, end in the lateral ocellar tract in the brain of the cricket and do not extend through the lateral ocellar nerve. The cockroach does not have an external median ocellus. Just as in the cricket, the cockroach also has only one soma where the locust has a two-cell cluster, L4–5.

Table 1. Animals with duplications and deletions of large ocellar interneurons.

Type of animal	Number examined	Interneurons		
		ML1–2	L1–3	L4–5
Animals with duplications				
Population A	45	8	1	1
Clutch A1	24	6	0	0
Clutch A2	24	0	0	0
Population B	45	1	13	1
Clutch B1	24	0	5	0
Clutch B2	24	0	5	1
Clutch B3	24	0	4	0
Normal ♂ × ML1–2 ♀*	24	7	1	0
Normal ♂ × normal ♀	24	1	6	0
Clone 2	42	1	22	2
Clone 4	12	0	6	0
Clone 6	24	0	1	0
Clone 7	24	0	1	0
Clone 8	24	0	0	0
Clone 9	18	1	6	0
Clone 10	2	0	0	0
Clone 12	6	0	0	0
Clone 13	6	0	0	0
Clone 16	8	0	0	0
Clone 17	6	0	0	0
Animals with deletions				
Clutch A1	24	0	6	0
Clutch A2	24	0	0	0
Clutch B1	24	0	0	0
Clutch B2	24	0	0	0
Clutch B3	24	0	0	0
Clone 2	42	0	0	0

*The female of this mated pair had a duplication of ML1–2.

The locust, *Schistocerca nitens*, has three simple eyes (ocelli) in addition to its large compound eyes. The axons of the ocellar interneurons extend through the three ocellar nerves (two lateral and one median) and have extensive arborizations in the peripheral ocellar neuropils. Each nerve normally contains the axons of seven large interneurons whose somata are located in the brain (Fig. 1A) (*6*). Both the somata and arborizations in the brain of the interneurons from one or more ocelli can be stained easily and repeatedly with cobalt (*6*), so that one can routinely examine the anatomy of large numbers of cells in large numbers of animals.

Supernumerary (duplicate) neurons of these interneurons were occasionally observed in a previous study of 50 animals (*3*). In the present study, the 14 interneurons whose axons run in the two lateral ocellar nerves were examined in 430 animals. Duplications were detected by the occurrence of four cells in the normal three-cell cluster, L1–3 (Fig. 2, A and B); three cells in the normal two-cell cluster, ML1–2 (Fig. 2, D to G); and three cells in the normal two-cell cluster, L4–5. When such extra cells occurred, they were apparently identical to their "twins" (Fig. 2H), so that the "true" cells were indistinguishable from the "extra" cells.

The possible genetic basis and specificity of these duplications were tested by selective breeding experiments. All animals were raised in nearly constant and identical conditions; caging conditions, crowding, feeding, temperature (31° ± 2°C), humidity (50 ± 5 percent), light and dark cycle (LD 16 : 8), and incubation of the eggs were all controlled.

Differences in the occurrence of duplicate cells were detected initially between two breeding populations of *Schistocerca nitens* (Table 1). Each population was founded from the eggs of a few closely related females taken from a separate and much larger breeding colony. Animals of population A had a tendency for duplications within one cell cluster (ML1–2), whereas animals of population B had a tendency for duplications within a different cell cluster (L1–3). These differences were maintained in the off-

spring of random clutches from unknown parents within these populations (Table 1, clutches A1, A2, B1, B2, and B3). In the second series of experiments, newly molted and thus unmated adults were separated into mating pairs in isolated cages. The adults were examined after the female had laid a clutch of eggs (80 to 100 eggs). In two cases, at least 24 male offspring of parents of known phenotypes were reared to adulthood and subsequently examined (Table 1). In the first case the offspring had a tendency for the same duplication (ML1–2) that had occurred in their mother (their father was normal); in the second case the offspring had a tendency for duplications within a different cell cluster (L1–3), although both parents were phenotypically normal.

While such results from breeding populations and single mated pairs demonstrate some degree of (i) genetic control and (ii) specificity of neuron duplications, the data are difficult to assess owing to the genotypic differences resulting from sexual reproduction. This problem of genotypic variability is eliminated in the case of animals from isogenic clones of locusts. The anatomy of identified neurons has previously been examined by serial-section reconstructions in small numbers of animals from within single clones in the water flea, *Daphnia magna* (*7*); the tropical fish, *Poecilia formosa* (*8*); and the nematode, *Caenorhabditis elegans* (*9*). Isogenic locusts differ from the previous examples in that they provide the opportunity to examine the anatomy (*10*) and physiology (*11*) of large and accessible identified neurons in large numbers of animals from many different clones.

Clones of isogenic locusts were produced by parthenogenetic breeding from two species: *S. nitens* and *S. americana* (*12*). Female locusts were induced to lay unfertilized eggs (*13*). The unfertilized eggs undergo meiosis with normal recombination and reduction leading to haploid eggs. Diploidy is restored by the fusion of cleavage nuclei (*14*, *15*). The reduction division causes each haploid egg to be genotypically different while the fusion of cleavage nuclei results in homozygosity. The second-generation parthenogenetic offspring from any one first-generation parthenogenetic female are isogenic since a homozygous female produces genotypically identical haploid eggs.

The ocellar interneurons were examined in 11 clones (Table 1). The results indicate that neuron duplications (i) can have a high degree of genetic control, (ii) can be highly specific, and (iii) can occur with a random, bilateral asymmetry. The genetic control of neuron duplications is shown by the large interclonal differences; the number of animals with duplications within one cell cluster (L1–3) ranged from 52 percent in clone 2 to 0 percent in clone 8. The high specificity of these duplications is shown by the occurrence of duplications within a particular

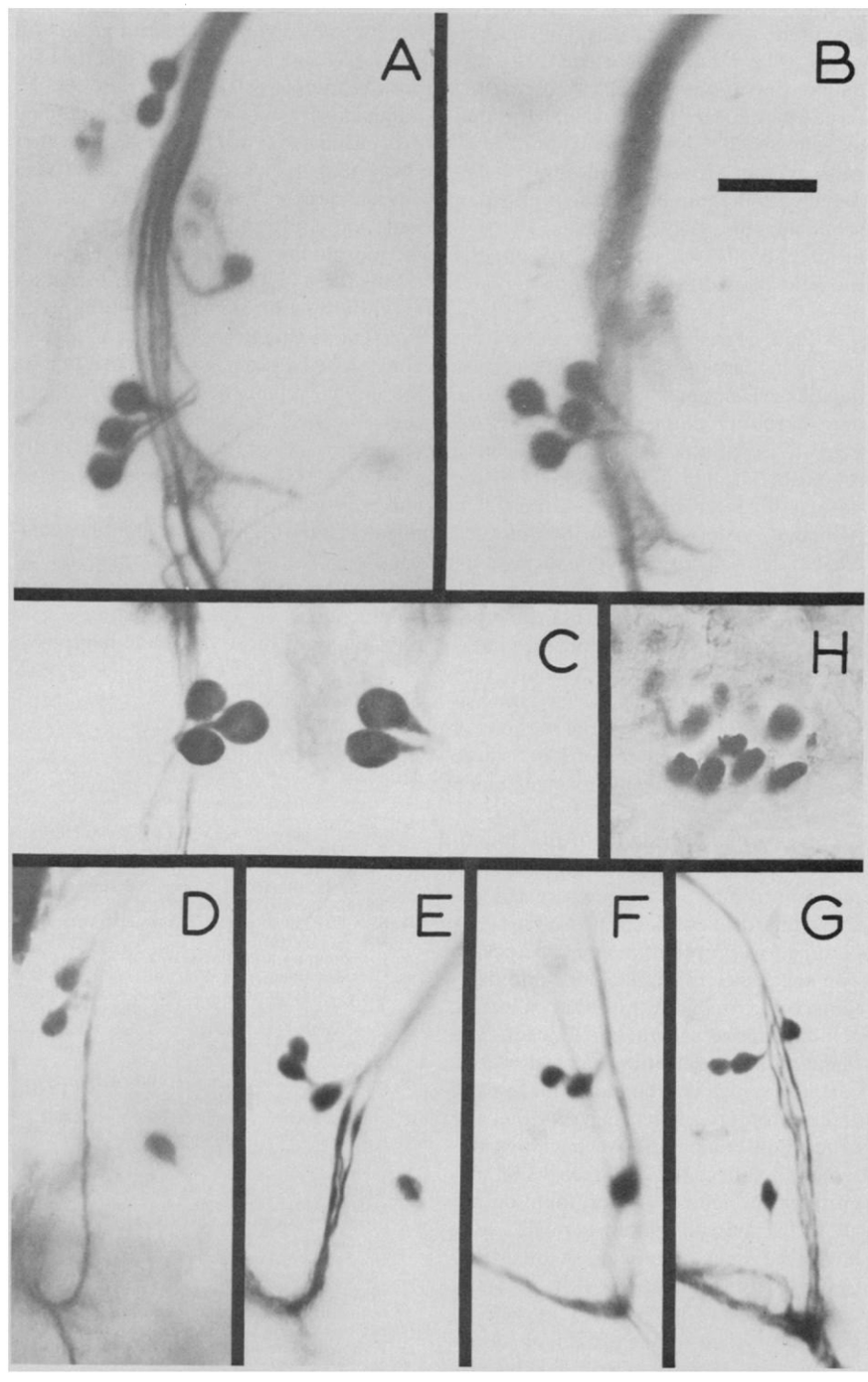

Fig. 2. (A to G) Photographs of whole-mounted brains of the locust, *Schistocerca nitens*, viewed posteriorly (A to E) and laterally (F and G), whose right lateral ocellar nerve (A and B), left and right lateral ocellar nerves (C), and median ocellar nerve (D to G) have been stained with cobalt dye. A duplication of the normal three-cell cluster, L1–3 (A), is shown by the occurrence of four cells (B). A deletion of L1–3 is shown by the occurrence of two cells (C). A duplication of the normal two-cell cluster, ML1–2 (D, F), is shown by the occurrence of three cells (E, G). Other somata out of focus or not shown in (A) and (B) are ML1–2 and L4–5; the soma below those of ML1–2 in (D to G) is M1. A cobalt injection of the left and right lateral ocellar nerves normally stains four axons extending through the median ocellar tract and nerve to the median ocellus ($ML1–2_L$ and $ML1–2_R$). In a Timm's silver intensification of a 15-μm section from such a cobalt injection in an animal with a duplication of ML1–2, a fifth axon is seen extending out the median ocellar tract (H); the supernumerary neuron is a complete anatomical duplicate of the normally occurring cells. [Scale bar: (A to C) 100 μm; (D to G) 86 μm; (H) 20 μm.]

cluster without any correlation with duplications of other interneurons examined. Although duplications within a particular cluster may occur unilaterally as well as simultaneously on both sides of the brain, this bilateral asymmetry appears to be random. In clone 2, for example, the number of cells in the normal three-cell cluster, L1–3, was distributed as follows: 20 animals, 3/3 (number of cells on the left side of the brain compared with the number on the right side); seven animals, 4/3; six animals, 3/4; seven animals, 4/4; two animals, 4/5; none of the animals had the distribution 5/4 or 5/5.

A high degree of genetic control and specificity can also be shown for neuron deletions. Deletions were not detected in any of the 11 clones examined. However, 25 percent of the animals in clutch A1 (Table 1) had only two cells in the normal three-cell cluster, L1–3 (Fig. 2C). Although it is possible that the third cell existed but was not stained in these preparations, this alternative seems unlikely because in all of the animals of the other clutches and clones all of the interneurons were completely stained. Furthermore, in Timm's silver intensifications of cobalt (*6*) in serial sections of two brains with deletions of L1–3, there were no large and conspicuous unstained somata near the two stained somata.

It has been suggested (*16*) that identified interneurons and motoneurons in the central nervous system are the descendents of identifiable neuroblasts according to a preprogrammed cell lineage. The specificity of duplications and deletions could result from either selective cell division or selective cell death. The finding that duplications and deletions can have a high degree of genetic control and neuron specificity suggests that the genetic differences between clones differentially affect the cell lineages of specific central neurons. Why, then, do not all of the animals within a single clone have the same number of neurons? Clones with duplications (for example, clone 2) show much more intraclonal variability than clones without duplications (for example, clone 8). One hypothesis is that the genotype of clone 2 is at the threshold level, given the "developmental noise," for the occurrence of a particular extra cell division (or absence of cell death). Extensive selective breeding might select for a genotype which, when cloned, would result in duplication of a neuron in all, rather than only 52 percent, of the animals.

Species-specific differences in the number of cells comprising apparently homologous groups of neurons have been found, for example, in the ocellar interneurons of locusts, crickets (*17*), and cockroaches (Fig. 1); in the dorsal-longitudinal motoneurons of crickets and cockroaches (*18*); and in the salivary effector neurons in the buccal ganglia of *Helisoma* and *Ariolimax* (*19*). Here I report genotypic variability expressed as duplications and deletions of identified neurons in an invertebrate brain; another study (*20*) shows genetic variability in neuron number in a vertebrate brain. Genetic variability has also been found in the morphology (*10*) and physiology (*11*) of identified neurons in concurrent studies with isogenic locusts. The differences in identified neurons between species could arise by natural selection acting on these types of genetic variability within a species. Just as modern hemoglobin chains are thought to have arisen by duplications of a common precursor chain and subsequent modification of the duplicate chains (*21*), so new identified neurons could arise by the duplication of preexisting neurons and the subsequent modification of the morphology and physiology of these duplicate neurons.

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References and Notes

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10. Isogenic locusts have been used to examine genetic variability in the morphology of identified ocellar interneurons (C. S. Goodman, in preparation).
11. Isogenic locusts have also been used to examine genetic variability in the effects of temperature of the neuronal threshold of identified thoracic motoneurons [C. S. Goodman and W. J. Heitler, *J. Comp. Physiol.* **117**, 183 (1977)].
12. These two closely related species have the identical number and morphology of large ocellar interneurons, and similar neuronal duplications can be found in both species.
13. Although I know of no cases of locusts breeding parthenogenetically in the field, female locusts can be induced to lay unfertilized eggs in the laboratory by crowding them and rearing them in close proximity to, but out of direct contact with, adult males. Male pheromones evidently induce sexual maturity and oviposition even in the absence of copulation [O. Okelo, thesis, University of California, Berkeley (1975)].
14. The few animals that hatch from unfertilized eggs (10 to 30 percent at most of the 50 to 100 eggs in a clutch) are diploid females [A. G. Hamilton, *Nature (London)* **172**, 1153 (1953)]. Since meiosis leads to a haploid egg (*15*), the important question is which of three genetic mechanisms is used to restore diploidy: (i) random fusion of a polar body nucleus with the egg nucleus or another polar body nucleus, (ii) selective fusion of the second polar body nucleus with the egg nucleus, or (iii) fusion of cleavage nuclei. To test this, a single-gene recessive mutant (non-sex-linked) of *S. nitens*, known as *tb* (having turquoise blue larvae) was obtained (from S. Hall, Zoecon); the homozygous mutant was crossed with a homozygous wild-type locust (having green larvae), and the heterozygous (+/*tb*) females were induced to lay eggs parthenogenetically. If (i) were the mechanism, the ratio of green (+/+ and +/*tb*) to blue (*tb*/*tb*) offspring would be 5 : 1, whereas (ii) and (iii) would lead to ratios of nearly 1 : 1, (ii) deviating in the 1+ : 1− direction according to the unknown rate at which crossing-over produces heterozygotes. The ratios of two such parthenogenetic clutches were 8 : 13 and 11 : 9, discounting mechanism (i). Two lines of evidence from other grasshopper species suggest that mechanism (iii) is used in those Orthoptera (the suborders Caelifera and Ensifera) in which parthenogenesis is facultative. This omits, for example, the acridid, *Warramba* (formerly *Moraba*) *virgo*, in which females normally reproduce by obligatory parthenogenesis in the field by means of a different and more successful genetic mechanism that does not involve the reduction to a haploid egg [M. J. D. White, J. Cheney, K. H. L. Ken, *Aust. J. Zool.* **11**, 1 (1963)]. The first line of evidence for fusion of cleavage nuclei comes from the studies of King and Slifer (*15*) who examined parthenogenetic eggs and embryos in several genera of grasshoppers and produced cytological evidence for fusion of cleavage nuclei. M. J. D. White [*Animal Cytology and Evolution* (Cambridge Univ. Press, Cambridge, ed. 3, 1973)] surveys the cytological literature on acridids and tetrigids and comes to the same conclusion. However, the best evidence is in tetrigid, *Paratettix texanus*. J. B. S. Haldane [*J. Genet.* **10**, 17 (1920)] showed that a number of multiple allelomorphs of *P. texanus* (*Hm*, *M*, and many others) were on the same chromosome as a factor Θ (in spermatogenesis the crossover value is about 24 percent while in oogenesis it is about 46 percent). We can reinterpret the data of R. K. Nabours and M. E. Foster [*Biol. Bull. (Woods Hole, Mass.)* **56**, 129 (1929)] as showing mechanism (iii), because, for example, their "item 12" shows an Hm+/MΘ female giving rise parthenogenetically to three Hm+/Hm+, five MΘ/MΘ, four HmΘ/HmΘ, and three M+/M+ female offspring. Only mechanism (iii) would lead to all crossovers being homozygous as in the last two sets of offspring. To prove conclusively that mechanism (iii) occurs in *Schistocerca*, one would have to use two single-gene mutations on the same chromosome. While the available evidence suggests mechanism (iii), the remaining alternative, mechanism (ii), would lead to isogenicity within several generations because the small amount of heterozygosity resulting from crossing over (the only cause of differences between the egg nucleus and the second polar body) is driven to homozygosity with each successive generation. The results described herein are from second to seventh generation parthenogenetic offspring; no change was observed in the percentage of clone 2 animals with duplications of L1–3 during these successive generations. The generation time is 3 to 4 months.
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22. I thank C. H. F. Rowell, D. Bentley, B. Mulloney, and W. Heitler for critical reading of the manuscript, O. Okelo for advice on grasshopper parthenogenesis, S. Hall for the *tb* mutant, and D. Moss for many animals used in these experiments. Supported by NIH grant NS 09404-05 to C. H. F. Rowell and NSF predoctoral fellowship to C.S.G.

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