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From epithelium to neuroblasts to neurons: the role of cell interactions and cell lineage during insect neurogenesis

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The grasshopper central nervous system is composed of a brain and a chain of segmental ganglia. Each hemiganglion contains about 1000 neurons, most of which can be individually identified by their unique morphology and synaptic connectivity. Shortly after gastrulation the ventral ectoderm becomes a neurogenic region. In each hemisegment, *ca.* 150 neurogenic ectodermal cells (nECs) give rise to a stereotyped pattern of 30 identified neuroblasts (NBs, neuronal stem cells); the remaining nECs become various non-neuronal cells or die. The 30 NBs then give rise to about 1000 neurons as each NB initiates an invariant lineage, generating a stereotyped chain of ganglion mother cells (GMCs), each of which in turn divides once to generate two identified neurons. We have used a laser microbeam or microelectrode to ablate individual cells *in ovo* and *in vitro* at various stages of embryogenesis to study how neuronal diversity and specificity are generated during development. Our results suggest (i) that cell interactions between *ca.* 150 equivalent nECs allow 30 cells to enlarge into NBs, the dominant fate in a hierarchy; (ii) the NBs inhibit adjacent nECs and thus cause them to differentiate into various non-neuronal cells; (iii) each NB is assigned its unique identity according to its position of enlargement within the neurogenic epithelium; (iv) each NB then generates its characteristic chain of GMCs by an invariant cell lineage; and (v) each GMC generates a pair of equivalent progeny, the fate of each individual neuron being determined by both its GMC of origin and interactions with its sibling.

Introduction

During grasshopper embryogenesis, an undifferentiated two-dimensional epithelial sheet is transformed into a highly differentiated three dimensional central nervous system. How this happens is the topic of this review. Pattern formation of the insect central nervous system during embryogenesis involves two major stages. In the first stage, a relatively uniform two-dimensional epithelial sheet develops into a stereotyped array of neuroblasts (NBs, neuronal stem cells) and non-neuronal cells. In the second stage, each NB within this array divides asymmetrically to generate a characteristic chain of progeny, called ganglion mother cells (GMCs), which in turn divide once more symmetrically to generate pairs of sibling cells (which differentiate into neurons). Thus, each NB contributes an essentially one-dimensional string of paired progeny which transforms the two-dimensional plate of NBs into a three-dimensional central nervous system.

Before considering the possible mechanisms underlying these developmental events, it is important to describe in some detail the development of both the NB pattern and NB lineages. The grasshopper central nervous system is composed of a brain and a chain of segmental ganglia. Each hemiganglion contains about 1000 neurons, most of which can be individually identified by their unique morphology and synaptic connectivity. Development of these neurons is initiated shortly after gastrulation as the midventral strip of ectoderm becomes a neurogenic

region. In each hemisegment, *ca.* 150 neurogenic ectodermal cells (nECs) give rise to a stereotyped pattern of 30 identified NBs (figure 1*a*); the nECs that do not become NBs instead differentiate into various non-neuronal cells or die (figure 2). Each NB generates a stereotyped chain of from 3 to 50 GMCs and then dies; each GMC divides once to generate a pair of sibling identified neurons. In this way, NBs generate characteristic families of from 6 to 100 neurons (see, for example, figure 1*b*). In summary, the pattern unfolds as *ca.* 150 epithelial cells generate

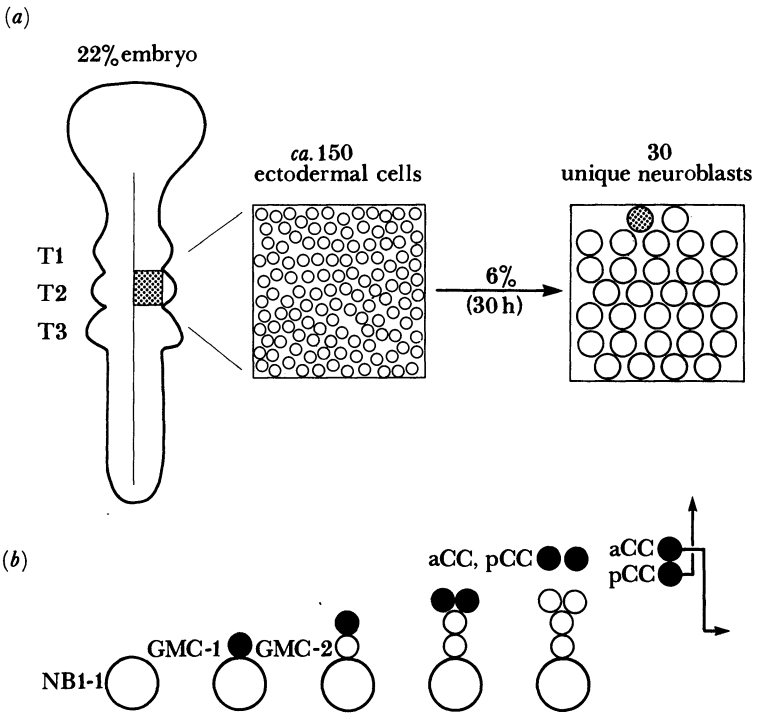

FIGURE 1. Schematic drawing showing the two major steps in grasshopper neurogenesis. (*a*) The first step is the transformation of a sheet of ectodermal cells into a stereotype pattern of unique neuronal precursor cells (neuroblasts; NBs). After gastrulation, the midventral strip of ectoderm becomes the neurogenic region, with about 150 cells per hemisegment; the mesothoracic (T2) hemisegment is shaded in the embryo (left) and enlarged (middle) to illustrate the uniformity and random arrangement of the ectodermal cells. Within 30 h (6% of embryogenesis) these 150 cells develop into a stereotyped pattern of 30 NBs; the remaining ectodermal cells form non-neuronal support cells surrounding each NB, or die. (*b*) The second step of neurogenesis is the production of a characteristic, invariant, family of neurons from each NB. Each NB divides asymmetrically to generate a chain of ganglion mother cells (GMCs), which divide once symmetrically to produce a pair of post-mitotic neurons. The lineage of NB 1–1 (shaded in (*a*)) is illustrated; the identified aCC and pCC neurons are always derived from the first GMC (GMC-1) of NB 1–1, and are used here as indicators of NB 1–1 development (see Goodman *et al.* 1984).

30 NBs; these NBs then produce *ca.* 500 GMCs, which divide into *ca.* 1000 neurons. What is most remarkable is that although the epithelial sheet appears undifferentiated and uniform, the NBs, GMCs, and neurons are all uniquely identified cells. Thus, the problem of how neuronal diversity and specificity is generated can be reduced to two more accessible questions, as discussed below.

First, how does a seemingly uniform epithelial sheet generate a highly organized pattern of unique NBs? Several alternative hypotheses could be proposed to answer this question (figure 3). (i) The process could be governed by a highly invariant cell lineage (figure 3*a*), such as in the nematode (see, for example, Sulston & Horvitz 1977; Sulston *et al.* 1983). This alternative

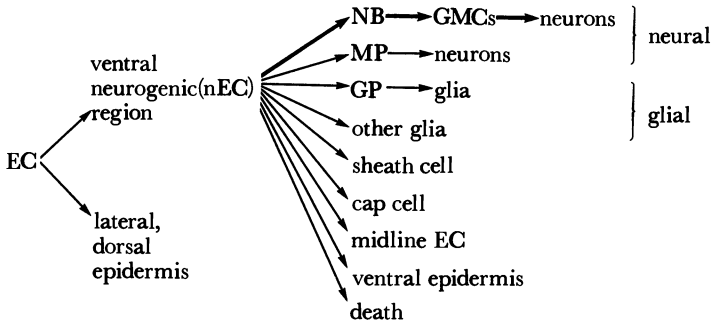

FIGURE 2. Diagram of the developmental fates assumed by ectodermal cells (EC) of the post-gastrula grasshopper embryo. The lateral and dorsal ECs produce non-ventral epidermis, whereas the mid-ventral ECs form a neurogenic region composed of neurogenic ECs (nECs) which produce neuronal precursors (neuroblasts, NB; and midline precursors, MP), glial precursors (GP), or differentiated non-neuronal support cells such as sheath cells, cap cells, or midline ECs. Some of these cells, or other nECs, will contribute to the ventral epidermis. In addition, nECs often degenerate during neurogenesis. See text for details.

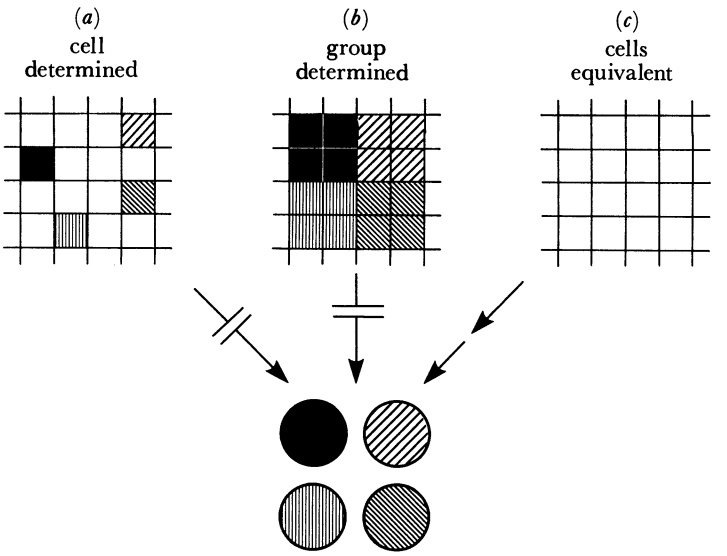

FIGURE 3. Three possible mechanisms of NB determination. nECs are represented as small squares; NBs derived from these nECs are represented below as circles. Shading of nECs indicates a restriction in fate to the similarly shaded NB. (*a*) 'Early determination'. Individual nECs are determined early in development to produce specific NBs. (*b*) 'Equivalence Groups'. Groups of nECs are restricted to form a specific NB and its support cells; interactions among the cells within a group allows only one cell to enlarge into the NB, the rest forming non-neuronal support cells around the NB (not shown). These theories, (*a*) and (*b*), are not supported by our data. Rather our results support theory (*c*) 'Cell interactions'. Initially all nECs are equivalent; a positional mechanism triggers local interactions among nECs (first arrow), and may provide the information for NB fate specification. The local cell interactions result in only one cell forming an NB (second arrow).

would imply that from early in development, specific cells within the epithelial sheet are uniquely committed to become specific NBs (figure 3*a*). (ii) The epithelium could be subdivided into equivalence groups (see, for example, Sulston & White 1980; Kimble 1981; Kimble & White 1981). Each equivalence group could thus be committed to generate a specific NB (the dominant fate) and its non-neuronal support cells, with the fate of each cell within the group being specified by cell interactions (figure 3*b*). (iii) The process could be governed entirely by cell interactions within a sheet of equivalent epithelial cells (figure 3*c*), as proposed for the

formation of chaeta in insects (see, for example, Wigglesworth 1940; Richelle & Ghysen 1979). All three alternatives can be directly tested by single cell ablation experiments.

Second, how does each NB generate its characteristic family of neurons? The same three alternatives equally apply to this question. Each neuron could be uniquely determined entirely by its lineage from a specific GMC and NB. Alternatively, each neuron could be determined by both its lineage and interactions within an equivalence group. Finally, each neuron could be determined entirely by positional interactions. Here too all three alternatives can be directly tested.

In this paper we review experiments in which a laser microbeam (or microelectrode) is used to ablate individual cells *in ovo* and *in vitro* at various stages of grasshopper embryogenesis (Doe & Goodman 1985*a*, *b*; Kuwada & Goodman 1985). Our results demonstrate the combined roles of cell interactions and cell lineage in generating neuronal diversity and specificity in the insect central nervous system. During the first stage, cell interactions between the *ca.* 150 equivalent nECs in a hemisegment allow 30 cells to enlarge into NBs, the dominant fate in a hierarchy. The NBs inhibit adjacent nECs and thus cause them to differentiate into various non-neuronal cells. Each NB appears to be assigned its unique identity according to its position of enlargement within the neurogenic epithelium. During the second stage, each NB generates its characteristic chain of GMCs by an invariant cell lineage. Each GMC divides once to generate a pair of equivalent progeny, the fate of each individual neuron being determined by both its GMC of origin and interactions with its sibling.

Identified neuroblasts beget identified neurons

Two types of neuronal precursor cells give rise to the neurons in the segmental ganglia of insects: neuroblasts (NBs; Wheeler 1893; Bate 1976) and midline precursors (MPs; Bate & Grunewald 1981). Neuroblasts are stem cells that divide repeatedly in an asymmetrical fashion, as described above, whereas MPs divide symmetrically only once to produce a pair of sibling neurons, much like GMCs. For example, the neuronal precursor cells in the mesothoracic or metathoracic segments include two symmetrical plates of 30 NBs each (figure 1) organized in seven rows of from two to five NBs per row, one median NB (MNB) at the posterior edge, and seven MPs along the dorsal midline. However, although most of the suboesophageal (S1–3), thoracic (T1–3), and abdominal (A1–10) segments are similar, not all have identical NB patterns (table 1; Doe & Goodman 1985*a*). The first seven abdominal segments contain only 29 NBs in each hemisegment (they are missing NB 5–5). Some segments (for example, T1) have an extra MNB located anteriorly, and others still are missing particular MPs. Moreover, the partial segments at the far anterior (S0) and posterior (A11) ends have dramatically altered patterns (table 1).

Each of these precursor cells can be individually identified according to its time of formation and position within the array, and according to the identified neurons that it generates (see, for example, Goodman & Spitzer 1979; Goodman 1982; Goodman *et al.* 1982; Raper *et al.* 1983; Taghert & Goodman 1984). Many of our experiments focus on a specific lineage: NB 1–1 generates GMC–1 which divides into a pair of sibling cells which differentiate into the identified aCC and pCC neurons (figure 1*b*; Goodman *et al.* 1982; Goodman *et al.* 1984). These two neurons have many cell-specific characteristics including their distinctive cell body location and axonal morphology. Their growth cones fasciculate with other axons in a highly specific

and stereotyped way; the pCC joins the MP1–dMP2 fascicle whereas the aCC joins the U fascicle. NB 1–1, GMC-1, and the aCC and pCC neurons are all highly accessible and easily identified, thus providing excellent assays for many of the cell ablation experiments described below (Kuwada & Goodman 1985; Doe & Goodman 1985*a*, *b*).

Table 1. Segmental differences in neuronal precursor cells

segment	number of NBs per hemisegment	NB 5–5	MNB	aMNB	number of MPs per segment	MP0
S0	13	–	+	–	0?	–
S1	24	–	+	+	4	–
S2,S3	29	–	+	–	7	–
T1	30	+	+	+	7	–
T2,T3	30	+	+	–	7	–
A1–A7	29	–	+	–	7	–
A8	30	+	+	–	8	+
A9	29	–	+	+	7	–
A10	25	–	+	+	7	–
A11	6	–	–	–	0?	–

Differentiation of neuroblasts

During development, the nECs in each hemisegment differentiate into many different cell types, or in some cases die (figure 2). We focus our attention here on the development of NBs and their support cells: cap cells (Kawamura & Carlson 1962), which tightly cap the ventral surface of each NB; and sheath cells, which surround each NB and extend end feet to both the dorsal and ventral surfaces of the neuroepithelium (figure 4). Each NB, its cap cell, and the adjacent sheath cells differentiate concurrently from a group of 4–8 nECs. All NBs do not develop simultaneously. While some groups of nECs are differentiating into early arising NBs and their sheath and cap cells, other nECs continue to divide, resulting in an expansion of the neurogenic region.

The first sign of NB differentiation from an nEC is the dorsal migration of the nucleus and cytoplasm (figure 4*b*). Occasionally more than one cell in a group begins to shift dorsally, but ultimately only one differentiates into an NB. As one of the nECs becomes an NB, further changes occur. The cell delaminates dorsally, the nucleus assumes a bilobed shape, and the cell enlarges from its initial diameter of 5–10 μm to a diameter of 20–30 μm (figure 4*c*). Unlike NBs in *Drosophila* (Poulson 1950; Hartenstein & Campos-Ortega 1984), NBs in the grasshopper maintain their large size until they degenerate. Approximately 1% (*ca.* 5 h) after an NB has enlarged, it begins its stereotyped series of asymmetrical divisions producing a chain of smaller GMCs extending dorsally (figure 4*d*).

The order and timing of NB formation is summarized in figure 5 (Doe & Goodman 1985*a*). NB formation is a continuous process. Invariably, NB 3–5 is the first NB to appear, followed by NB 2–5, and then by a group of eight NBs whose enlargement is virtually simultaneous (all ten of these NBs plus the next four we call the 'early' NBs). For simplicity, we have grouped the NBs into early, middle, and late groups as shown in figure 5. Clearly, the NB pattern does not form in either a simple anterior–posterior or medial–lateral fashion. Ultimately, all nECs differentiate into either NBs, their non-neuronal support cells, other non-neuronal cells, or die.

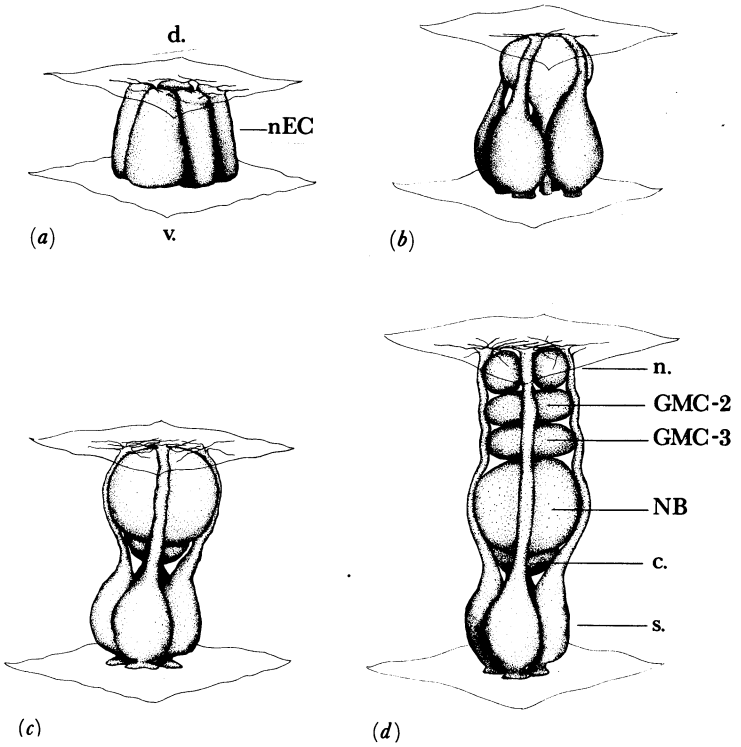

FIGURE 4. Morphological differentiation of neuroblasts and support cells in the neurogenic region. Drawings based on embryos viewed by transmission and scanning electron microscopy and Lucifer Yellow dye injections of single cells. Dorsal (d.) and ventral (v.) surfaces of the neuroectoderm are indicated. (*a*) A group of neurogenic ectodermal cells (nECs); filopodia extend from the dorsal surface of the cells. (*b*) One nEC has begun differentiating into an NB; the cytoplasm and nucleus have shifted dorsally and delamination of the cell from the ventral surface of the embryo has begun. (*c*) The new NB is fully delaminated from the ventral surface, has enlarged to 20–30 μm in circumference, and has few filopodia. The surrounding cells, formerly nECs, have differentiated into a cap cell (c.) closely apposed to the ventral surface of the NB (drawn smaller than scale for clarity); and sheath cells (s.) with cytoplasmic extensions ensheathing the NB and extending dorsally. The number of sheath cells around each NB can vary. (*d*) The NB has divided three times, generating three ganglion mother cells (GMCs). The first of the three GMCs (GMC-1) has divided into two post-mitotic neurons (n.). The second and third GMCs (GMC-2 and GMC-3) have yet to divide. Sheath cells continue to enwrap the NB and span the thickness of the neuroepithelium.

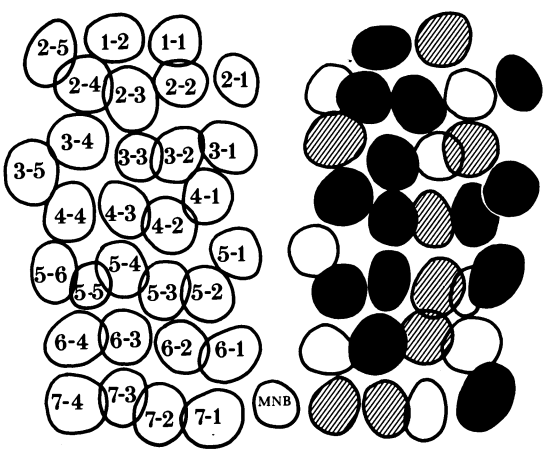

FIGURE 5. Temporal and spatial pattern of NB formation. Camera lucida drawing of a mature NB pattern; NB identities are indicated in the left hemisegment, time of formation of each NB is indicated by shading in the right hemisegment. Although each NB enlarges at a specific time, for simplicity only three groups are depicted: black NBs form first, cross-hatched NBs form second, and clear NBs form last. The early NBs form three columns; the middle NBs form a fourth column, filling in the space between the early NB columns. The late NBs form, for the most part, at the periphery of the pattern.

The temporal information germane for most of the experiments described below is that NB 1–1 appears fourth, NB 1–2 appears 21st (and therefore some 15 or more hours later than NB 1–1), and NB 7–3 appears last in the abdominal segments (in T1–3 and A8, the segment-specific NB 5–5 appears last). Thus, for many hours after NB 1–1 forms, a cluster of nECs lies in the adjacent 1–2 location. Furthermore, NB 7–3 forms last from a cluster of nECs that lies between NB 7–2 and NB 7–4 and is bordered by only differentiated NBs and support cells; no other nECs exist in this area by this time.

Neuroblast pattern is generated by cell interactions

When one of the cells within a cluster of nECs differentiates into an NB, the remaining cells either differentiate into non-neuronal support cells (sheath cells or cap cells) or die. To discover whether one specific nEC is determined to become a particular NB, we ablated one or more nECs in the 7–3 position both *in vitro* and *in ovo* (figure 6; test of hypothesis in figure 3*a*; Doe & Goodman 1985*b*). When only the enlarging nEC is killed (that is, the cell that is

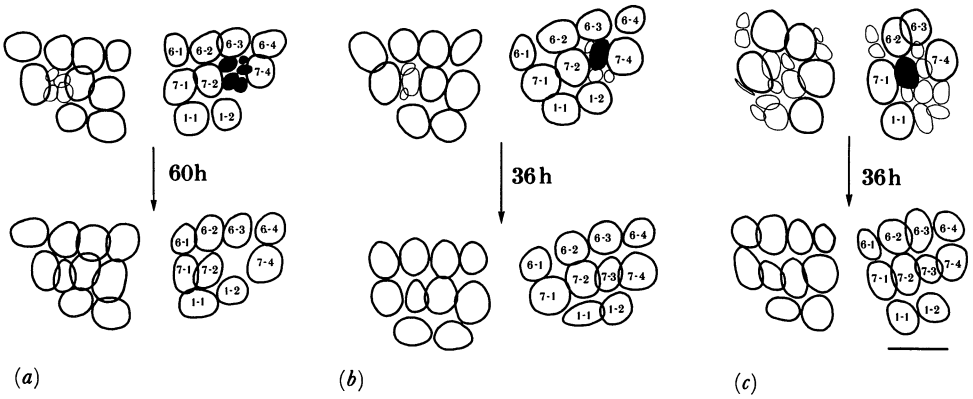

Figure 6. Individual nECs are not determined to form specific NBs. Camera lucida tracings of cells after *in vitro* laser ablations of nECs (top) and after further development in culture (bottom). The blackened cells were ablated. The numbered cells represent mature NBs and their support cells. (*a*) All nECs lying between NBs 7–2 and 7–4 were killed. On the control side (left) NB 7–3 developed after 24–36 h, whereas on the experimental side (right) NB 7–3 failed to develop even after 60 h of culture. (*b*) The presumptive NB 7–3 was killed and after 36 h of culture a new NB 7–3 developed from an adjacent nEC. (*c*) The presumptive NB 7–2 was killed before it began to divide; after 36 h of culture both NB 7–2 and NB 7–3 developed on the control and experimental sides. Scale bar: 50 μm.

differentiating into NB 7–3), a new NB 7–3 always forms (figure 6*b*). When all the nECs at the 7–3 position are ablated, NB 7–3 never develops (figure 6*a*). Thus more than one cell can become a particular NB. Furthermore, local inhibition of nECs by the enlarging NB allows development of only one NB in each group. Finally, in these experiments only the undifferentiated nECs can form NBs; adjacent NBs or their differentiated non-neuronal support cells cannot. Similar experiments have been performed on the enlarging NB 7–2 (figure 6*c*).

To test the generality of the conclusion that only nECs have the potential to form into NBs, and to find out if nECs at one NB position can replace an adjacent (different) NB, we performed a series of *in ovo* laser ablations (Doe & Goodman 1985*b*). The results of these experiments indicate that when the killed NB is adjacent to a cluster of nECs, the NB is typically replaced; when neighbouring nECs are absent, the NB is not replaced. Furthermore, only nECs can form NBs; lateral ECs cannot. For example, when NB 3–5, which lies on the lateral edge of the

neurogenic region, is ablated when no adjacent nECs remain (that is, after the adjacent medial NBs have enlarged and begun dividing), regulation never occurs, even though lateral ECs (from just outside the neurogenic region) are adjacent to the ablated NB. Moreover, this experiment indicates that the initial nECs in the 3–5 position have now all differentiated into non-neuronal support cells which cannot regulate to replace the ablated NB 3–5. The major conclusion of all of the above experiments is that only neighbouring nECs can regulate to replace ablated NBs.

Neuroblasts are determined by their position

It is clear that an EC from one position can enlarge to form an NB in an adjacent position. The next question is whether the replaced NB has the identity of the position from which the nEC originated (the donor position, indicating that the nECs are determined as a group to produce a specific NB; hypothesis B in figure 3), or alternatively the identity of the adjacent ablated NB in whose position it has enlarged (the host position, indicating that the nECs acquire a specific NB fate according to their position of enlargement; hypothesis C in figure 3).

When NB 1–1 is ablated, one of the adjacent nECs from the 1–2 position enlarges to form an NB in the 1–1 position. Thus, two NBs are produced from a single cluster of nECs: one in the initial position (1–2) and one in the new position (1–1). If position of enlargement determines NB fate, we would expect the regulated NB to have the 1–1 identity and produce the aCC and pCC neurons. The results of such experiments confirm this prediction; the regulated NB produces the aCC and pCC neurons (figure 7, 8*c*; Doe & Goodman 1985*b*). The only difference from the control side is that the aCC and pCC neurons from the regulated NB are delayed about 10 h, the time it takes for NB regulation to occur. These results show that groups of nECs are not determined to form specific NBs (figure 3*b*); rather the fate of the regulated NB is determined shortly after its enlargement, probably due to its position in the neuroectoderm.

Ganglion mother cells are determined by their lineage

Given that NBs are positionally determined within the two dimensional epithelial sheet, we wondered whether the NB progeny in the third dimension, the GMCs, are determined by position or lineage. We already had a good indication that the NB of origin was important; when NB 7–3 was permanently ablated (that is, later in development when no nECs remained to replace it), two of its characteristic progeny, the identified neurons S1 and S2, were not generated by any other NB (Taghert *et al.* 1984). Thus, the two sets of experiments described below were designed to test whether GMCs are determined not only by their NB of origin, but more specifically by their particular cell division of origin.

In the first set of experiments, we used a microelectrode to ablate GMC-1 from NB 1–1 *in vitro* immediately after its birth, but before GMC-2 had been born (Doe & Goodman 1985*b*). Cell assays revealed that GMC-2 (born about 5 h after GMC-1) did not regulate to replace GMC-1; the aCC and pCC neurons, normally derived from GMC-1, were absent (figure 8*e*). As described above, a regulated NB 1–1 will produce a normal GMC-1, and consequently the aCC and pCC neurons, up to 15 h later than normal. The fact that GMC-2 is born less than 5 h after ablation of GMC-1 and yet fails to make the aCC and pCC neurons suggests the control of GMC-1 fate by lineage rather than temporally regulated interactions.

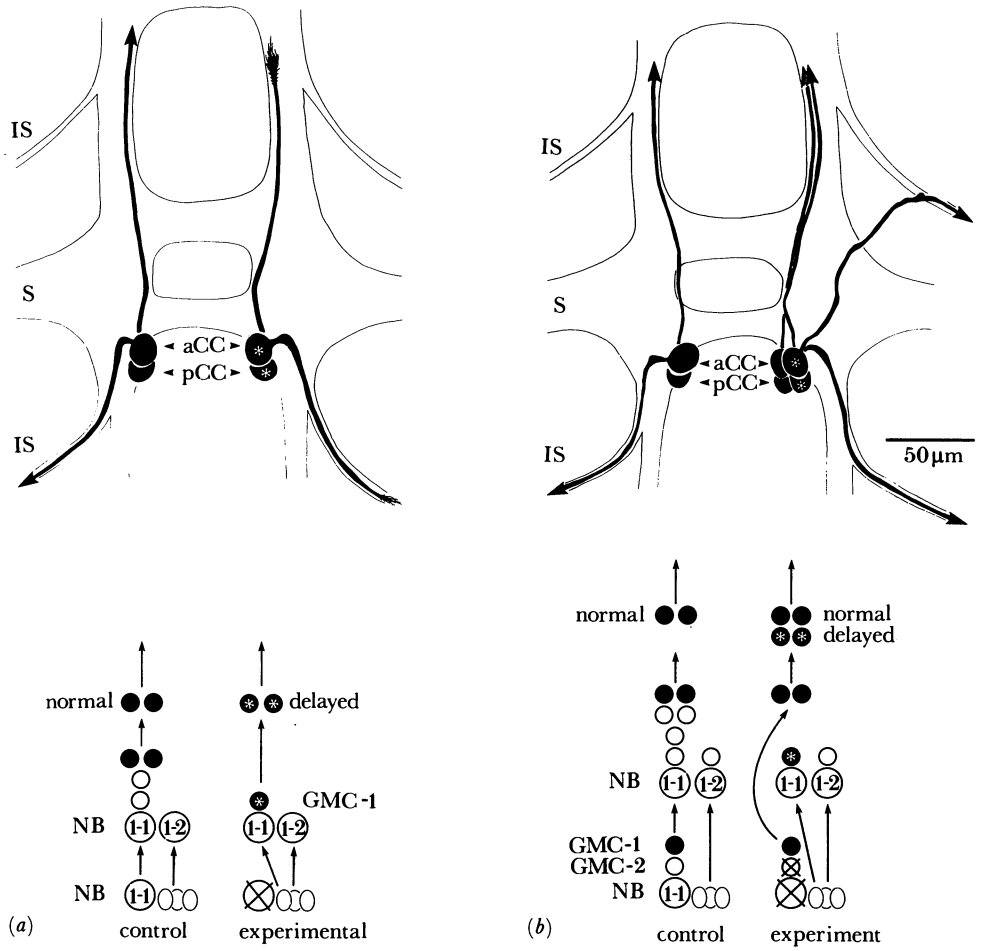

FIGURE 7. Regulation of neuroblasts and duplication of identified neurons. (*a*) The identity of a developing NB is apparently determined by its position of enlargement in the neuroectoderm. When NB 1–1 is ablated before it begins to divide, one nEC from the adjacent 1–2 position moves over and enlarges into an NB (shown schematically at the bottom of the figure). This new NB is shown to have the determination of NB 1–1 (the host position), rather than NB 1–2 (the donor position) by the development of aCC and pCC neurons (blackened with asterisk on the experimental side), which are derived from GMC-1 of NB 1–1. As expected, the aCC and pCC neurons on the experimental side are about 10 h delayed relative to the contralateral control aCC and pCC neurons, due to the time it takes for regulation of the ablated NB 1–1. (*b*) Duplicated aCC and pCC neurons produced by a regulated NB 1–1. Schematic at the bottom of the figure illustrates the ablation protocol: on the experimental side NB 1–1 was ablated (as was GMC-2) but GMC-1 (blackened) was left untouched. GMC-1 ultimately develops into normal aCC and pCC neurons (blackened), as does the GMC-1 in the control hemisegment. Regulation of the killed NB 1–1 occurs as an nEC from the adjacent 1–2 position enlarges into an NB. This new NB begin the NB 1–1 lineage anew, despite the presence of GMC-1 progeny from the ablated NB 1–1, as seen by the existence of a duplicate pair of aCC and pCC neurons (blackened with asterisk). The camera lucida (top of figure) shows only the proximal part of each neuron; their axons extend beyond the figure borders. Both pCCs extend anteriorly as normal in the MP1–dMP2 fascicle; the normal aCC follows its usual path out the intersegmental nerve (IS) of its own segment, whereas the duplicate aCC grows out the IS nerve of the adjacent anterior segment. S, segmental nerve. Thin line represents outline of neuropil region. Scale: 50 μm.

In the second set of experiments, we wanted to know whether a regulated NB would begin its lineage anew, even if the ablated NB had already divided, and despite the presence of progeny from the ablated NB. To answer this question, we ablated NB 1–1 after it had produced GMC-1 and GMC-2 (figures 7*b* and 8*d*). We assayed for duplicated aCC and pCC neurons. When regulation of NB 1–1 occurred, we observed duplication of the aCC and pCC neurons (figure 7*b*). The duplicated neurons were about 20 h delayed relative to their control contralateral and ipsilateral homologues. In all experiments, the duplicated pCC extended its growth cone anteriorly as normal along the MP1–dMP2 fascicle, whereas the duplicated aCC extended its growth cone anteriorly, instead of posteriorly as normal, and then laterally along the U fascicle from the next anterior segment. This departure from the aCC's normal pathway can be attributed to its 20 h delay in initiating axonogenesis, an interesting experimental result outside the scope of this review (see Doe *et al.* 1986). Taken together, these two sets of experiments indicate that GMCs are determined by their cell division of origin from a particular NB.

Neurons are determined by interactions and lineage

Having shown that GMC-1 from NB 1–1 is determined by its lineage, we wondered what causes its two progeny, the aCC and pCC neurons, to differ from one another (Kuwada & Goodman 1985). When either one of these two GMC-1 progeny is ablated within 5 h after their birth, the remaining cell differentiates into the pCC (figure 8*f*). However, when ablations are made between 5 and 10 h after their birth (yet still before their axonogenesis), the remaining cell becomes either the aCC or pCC with equal probability. These results suggest that the sibling progeny from GMC-1 are (i) initially equivalent, (ii) become uniquely determined by early interactions, and (iii) exhibit a hierarchy of fates whereby the pCC is dominant. The same results were obtained for a pair of sibling progeny produced by another precursor, MP3 (Kuwada & Goodman 1985). Thus identified neurons are determined by their lineage from a specific NB and GMC, and by their interactions with their sibling from the same GMC; the pairs of GMC progeny form equivalence groups within which cell interactions determine their ultimate fate according to a hierarchy.

Conclusions

At the outset of this review, we posed two questions. First, how does a seemingly uniform epithelial sheet containing about 150 cells per hemisegment generate a highly organized pattern of 30 NBs? Second, how does this stereotyped pattern of 30 NBs generate *ca.* 1000 identified neurons? We have shown that cell interactions control the first stage (Doe & Goodman 1985*b*), whereas cell lineage (Doe & Goodman 1985*b*) and cell interactions (Kuwada & Goodman 1985) the second. Each of these conclusions is discussed below.

Within the sheet of undifferentiated nECs, any cell can become an NB. Cell interactions between the *ca.* 150 equivalent nECs allow 30 cells to enlarge into NBs. Cell ablation experiments suggest that the NB fate is dominant in a fate hierarchy. Evidently, once a cell enlarges to become an NB, it inhibits the cells around it from becoming NBs (figure 9*a*, *b*); they then differentiate into various non-neuronal cells. Released from inhibition by NB ablation, one of the undifferentiated nECs in the vicinity becomes an NB (figure 9*c*, *d*); in contrast, the neighbouring differentiated non-neuronal cells cannot become NBs. While our results suggest

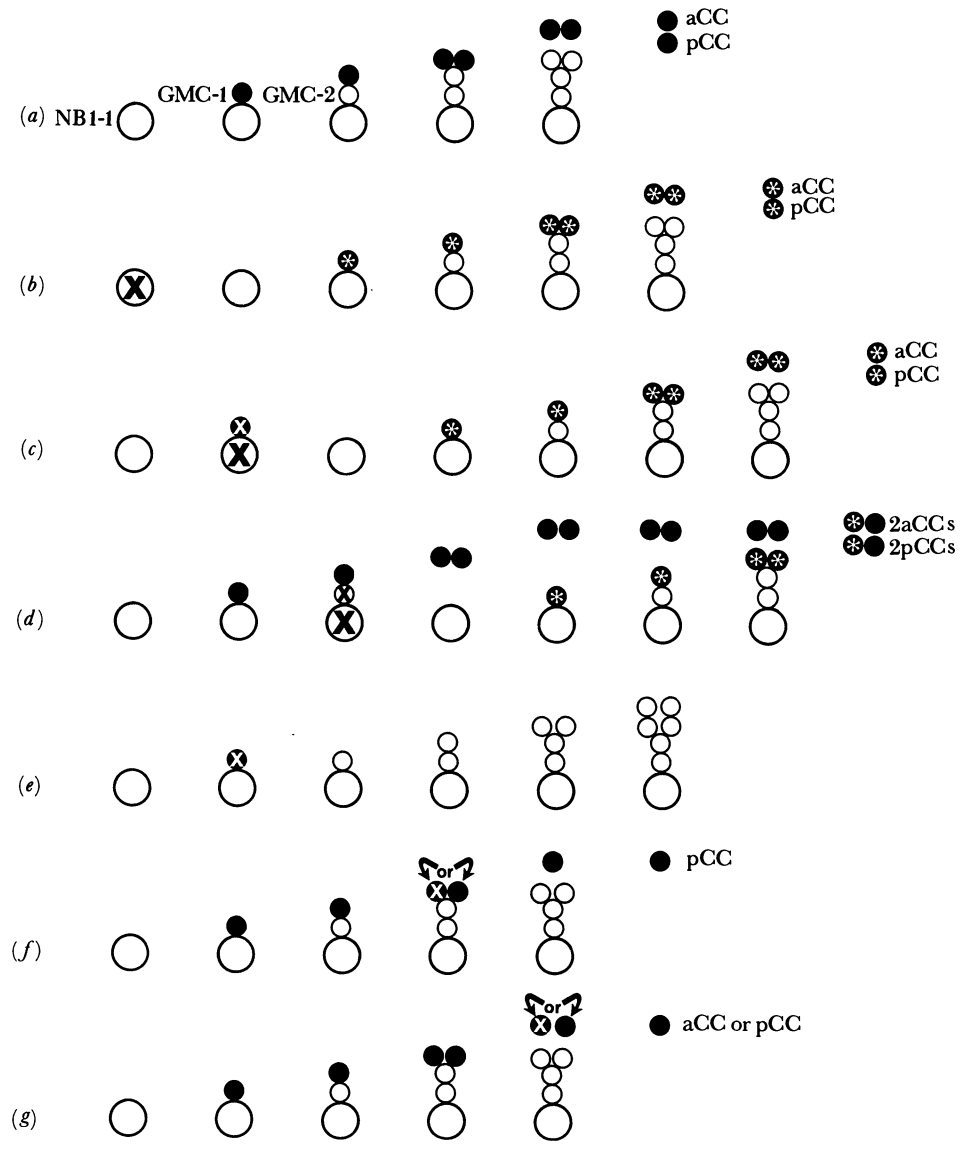

FIGURE 8. Cell lineage and cell interactions generating the aCC and pCC neurons. Developmental time goes from left to right in approximately 5 h intervals between drawings. The aCC and pCC, as well as their precursor GMC-1, are blackened; when derived from a regulated NB 1–1 these cells are marked with an asterisk (*). (*a*) Normal lineage of aCC and pCC neurons. (*b*) Ablation of NB 1–1. An adjacent nEC enlarges to replace the NB; it produces normal aCC and pCC neurons, despite being delayed up to 10 h. (*c*) Ablation of both NB 1–1 and GMC-1 results in regulation and the production of normal aCC and pCC neurons delayed 5 h more than those in B. (*d*) Duplication of aCC and pCC neurons following ablation of NB 1–1 and GMC-2, but not GMC-1. The original GMC-1 produces normal aCC and pCC neurons, whereas the regulated NB 1–1 begins the lineage anew and produces a second duplicate set of aCC and pCC neurons delayed 15–20 h relative to the normal aCC and pCC neurons. (*e*) Ablation of GMC-1 immediately after its birth; GMC-2 does not make aCC and pCC neurons, despite being born only 5 h later than GMC-1. (*f*) Ablation of either of the progeny of GMC-1 within 5 h of their birth. The remaining cell differentiates into the pCC neuron. (*g*) Ablation of either of the progeny of GMC-1 5–10 h after their birth; the remaining cell shows an equal probability of forming an aCC or pCC neuron. See text for discussion of these results.

that many, if not all, nECs are equivalent, we cannot rule out the possibility that boundaries exist within the *ca.* 150 nECs restricting their fate, for example, either at segment borders or at other putative compartment borders within a segment. We have only observed regulation of six NBs from five groups of neighbouring nECs, and it is only in these regions that we can be sure of nEC equivalence: from 1–2 to 1–1; from 3–4 to 3–5; from 3–3 to 4–4; from 7–2 to 7–1; from 7–3 to 7–2; and from 7–3 to 7–4.

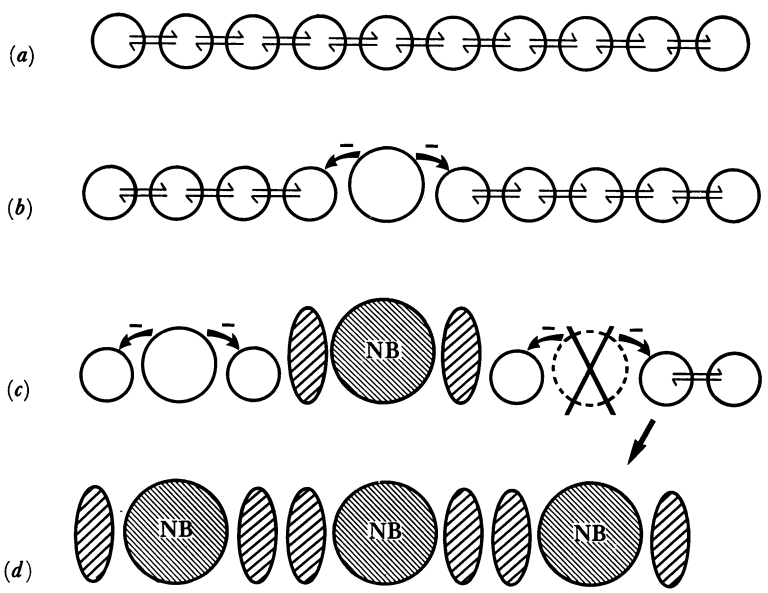

FIGURE 9. Schematic drawing illustrating the local inhibition of adjacent nECs by an enlarging NB. (*a*) All nECs are equivalent and interact with each other (half-arrows). (*b*) One cell begins to enlarge into an NB and inhibits the adjacent cells from enlarging (arrows). (*c*) The middle cells have differentiated (NB, narrow cross-hatching; support cells, wide cross-hatching); the lateral NBs have begun enlarging and inhibit the adjacent nECs (arrows). If an enlarging NB is ablated (dashed circle on the right), the adjacent nECs are released from inhibition and one will enlarge to replace it. (*d*) Ultimately all cells differentiate into either NBs (narrow cross-hatching) or non-neuronal support cells (wide cross-hatching).

Similar short range inhibition of cell differentiation has been proposed in both plants (Wilcox *et al.* 1973; Schoute 1913; Mitchison 1977) and animals (Wigglesworth 1940; Moscoso del Prado & Garcia-Bellido 1984). It is interesting that mechanisms proposed for the development of insect macrochaetae mother cells (Richelle & Ghysen 1979), which generate peripheral sensory neurons, can also be used to explain the development of insect neuroblasts, which generate central neurons, as discussed below. (i) Positional interactions within an epithelial sheet gives groups of cells in specific positions a high probability of forming an NB or chaeta mother cell. (ii) Cell interactions among one such group of cells results in one cell differentiating into an NB or chaeta mother cell. (iii) The differentiated cell inhibits its neighbours from undertaking an identical developmental pathway; these cells assume non–neuronal fates. (iv) Ultimately, a highly stereotyped pattern of NBs or chaetae is formed. An apparent difference is that, in contrast to the pattern of chaetae, the NB pattern is more densely packed and the observed inhibition confined to nearest neighbours. Interestingly, when the *scute* gene is 'derepressed' (Moscoso del Prado & Garcia-Bellido 1984), a greater number of macrochaetae are produced. Clonal analysis suggests that the density of macrochaetae mother cells at their

time of determination is 1 per 1–2 cell diameters (Moscoso del Prado 1982, cited in Moscoso del Prado & Garcia-Bellido 1984), quite similar to the density of NBs.

It appears that each NB is assigned its unique identity according to its position of enlargement within the neurogenic epithelium. An nEC can be 'transplanted' to an adjacent NB position by *in ovo* ablation of that NB. The nEC then enlarges in place of the ablated NB and differentiates into the positionally correct NB, producing neurons characteristic of the host rather than donor position. That each NB is uniquely determined to generate a specific family of neurons is clear. How such a pattern of 30 unique NBs is specified is totally unknown. One interesting observation is the discovery of positionally expressed antigens in the developing neurogenic region. Monoclonal antibodies (MAbs) have been generated which recognize subsets of nECs and NBs during the time the NB pattern is developing (Kotrla & Goodman 1982; C. Q. Doe, K. Kotrla & C. S. Goodman, unpublished results). The MAb EPI-1 binds to a cell surface antigen initially expressed in the nECs and NBs in rows 2 and 6 of each segment; as development proceeds it ultimately stains all of the nECs, NBs, and their support cells, although none of the NB progeny. MAb EPI-4 initially stains all nECs before neurogenesis, gradually becoming restricted to the cells of NB rows 1 and 5 before disappearing. Whether these antigens play any role in this complex pattern formation is unknown. They do, however, indicate that positionally expressed molecules exist during the period of neurogenesis, and that their spatial expression is dynamic.

Determined by its position, each NB goes on to generate its characteristic chain of GMCs by an invariant cell lineage. For example, our results indicate that GMC-1 from NB 1–1 is born intrinsically determined, that is, as a consequence of its lineage, rather than as a consequence of its spatiotemporal environment (Doe & Goodman 1985*b*). One of the best confirmations of this conclusion is the observation of duplicated neurons (figure 7). Even though the ablated NB 1–1 has already generated GMC-1, the regulated NB began its lineage anew. Although delayed some 20 h relative to normal, the regulated NB 1–1 nevertheless generates a new GMC-1, thus giving rise to two pairs of aCC and pCC neurons, both of which survive and differentiate.

Finally, each GMC generates a pair of equivalent progeny, the fate of each individual neuron being determined by both its GMC of origin and interactions with its sibling (Kuwada & Goodman 1985). The pairs of GMC progeny act as equivalence groups in which each of the two neurons becomes uniquely determined according to a fate hierarchy. For example, if GMC-1 from NB 1–1 is ablated, the aCC and pCC neurons never form, indicating that only the progeny from GMC-1 can become these two neurons. However, shortly after their birth, if either one of the two GMC-1 progeny is ablated, the remaining cell becomes the pCC neuron, indicating that pCC is the dominant fate. Thus, a complex pattern of both cell lineage and cell interactions is involved in determining each individual identified neuron.

Having completed a fairly detailed cellular analysis of insect neurogenesis, the next step is a molecular genetic analysis of these developmental events. Whereas the grasshopper embryo has been ideal for the cellular studies described here, the *Drosophila* embryo has obvious attributes for a molecular genetic approach. Fortunately, there exist many similarities between *Drosophila* and grasshopper in both the patterns of neuronal precursor cells (Hartenstein & Campos-Ortega 1984; Thomas *et al.* 1984) and the differentiation of their identified neuronal progeny (Thomas *et al.* 1984; Goodman *et al.* 1984; Bastiani *et al.* 1985). Certain *Drosophila* mutations already exist, called Notch-like mutants, which alter the pattern of NBs by increasing

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their number at the expense of the non-neuronal cells (see, for example, Lehmann *et al.* 1983). It seems likely that some of these Notch-like genes may be involved in the local inhibition from NBs to surrounding nECs discussed here, the mutation of such genes thus allowing many more nECs to differentiate into NBs.

But for some of the most important events of insect neurogenesis, the determination of the 30 unique NBs in each hemisegment and the lineal determination of their GMC progeny, there are presently no candidate genes. One hypothesis would predict that, just as the Bithorax and Antennapedia gene complexes control the determination of 14 segments (see, for example, Lewis 1978; Bender *et al.* 1983; Lawrence & Morata 1983), so an analogous set of neurogenic gene complexes might control the determination of the NBs in each hemisegment, and the subsequent determination of their GMC progeny. It will be interesting to see if this prediction holds true.

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