The Journal of Neuroscience, May 1993, 13(5): 2068–2084

Sodium Channels, $GABA_A$ Receptors, and Glutamate Receptors Develop Sequentially on Embryonic Rat Spinal Cord Cells

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It is not well understood when during embryonic development the elements of a cell's responsiveness first appear, nor the factors controlling their appearance. A strategy to approach this issue is to determine which aspects of neuronal development are highly stereotyped in presence, timing, or pattern across a variety of cell types, and which are more diversified by cell type, region, or other parameters. We have used a fluorescent potentiometric oxonol dye in conjunction with a digital video imaging system to record the emergence and distribution of specific forms of excitability in dissociated embryonic rat spinal cord cells. We studied the expression of responses to veratridine, a sodium channel activator; muscimol, a $GABA_A$ receptor agonist; and kainic acid, an agonist at a class of glutamate receptors. Responses were consistently detectable in a percentage of cells dissociated from the earliest age examined, embryonic day 13, and increased progressively in later ages. Cells were examined from four regions, with cervical-lumbosacral and ventrodorsal distinctions. In the population of cells from each region, functional sodium channels appeared prior to $GABA_A$ receptors, which in turn emerged prior to kainate-activated glutamate receptors. This pattern was common to all spinal cord regions and revealed ventrodorsal and rostrocaudal gradients reflecting the known pattern of spinal cord neurogenesis. Analysis of the individual cell responses indicated that the stereotypical pattern of sequential channel development occurs individually on most cells in each region.

[Key words: rat spinal cord, embryonic development, sodium channels, $GABA_A$ receptors, non-NMDA glutamate receptors, oxonol]

Membrane responsiveness to both chemical and electrical stimuli is essential for nervous system function. Many components of excitability develop during the embryonic period. Voltage-gated ion channels appear early in neuronal differentiation, including sodium channels (Shen et al., 1988; Beckh et al., 1989; McCobb et al., 1990), calcium channels (Baccaglini and Spitzer, 1977; McCobb et al., 1989), and potassium channels (Beckh and Pongs, 1990; McCobb et al., 1990). Receptors for a variety of substances are expressed during the embryonic period, including GABA, glycine, and excitatory amino acids (Bixby and Spitzer, 1982; Blanton et al., 1990; Koller et al., 1990; Mandler et al., 1990; LoTurco et al., 1991). Also appearing during this time are transmitters such as GABA, glutamate, and 5-HT (Wolff et al., 1984; Fuji et al., 1985; Bregman, 1987; Marti et al., 1987; Blanton and Kriegstein, 1991; Cobas et al., 1991) as well as peptides such as cholecystokinin, vasoactive intestinal peptide, somatostatin, and substance P (Fuji et al., 1985; Marti et al., 1987). In the mature CNS, these receptors and transmitters mediate synaptic transmission. However, many of these properties appear to occur prior to synaptogenesis (Shen et al., 1988; Blanton and Kriegstein, 1991; Cobas et al., 1991) and may have roles in early development other than that of the fast, transient, intercellular communication function classically attributed to neurotransmitters. Some of these neurotransmitters may function as trophic agents, influencing the survival (Moran and Patel, 1989; Brenneman et al., 1990) and subsequent development of the neurons upon which they act (Hansen et al., 1984; Lauder, 1987; Mattson, 1988; Meier et al., 1991; Spitzer, 1991). Some receptors may appear only transiently on a cell during development (Bixby and Spitzer, 1984; Ito and Cherubini, 1991), while others may have an initial molecular composition to be later replaced with a different form of the receptor embodying those properties found in mature cells (Poulter et al., 1992). These changes may also be seen in the voltage-gated ion channels (Beckh et al., 1989; McCobb et al., 1989; Beckh and Pongs, 1990). In more advanced stages of development, neuronal activity with synaptic transmission can have long-lasting effects on circuit formation (Shatz, 1990a,b). Many aspects of this development remain incompletely understood. One approach is to examine cell properties and determine to what degree a portion of development is stereotyped such that virtually all neurons proceed in the same manner, exhibiting the same pattern of development. The alternative is a more variable development of properties, reflective of the more varied nature of the eventual differentiated state.

The spinal cord can be a useful area for such investigations. Neurogenesis occurs with ventral-to-dorsal, and rostral-to-caudal overall gradients (Nornes and Das, 1974; Altman and Bayer, 1984). There are a variety of neuronal types, with a degree of physical separation between cell types feasible from very early stages of embryogenesis due to the ventrodorsal distinction of motor and sensory systems.

We have utilized a novel recording strategy to study the emergence and distribution of three forms of membrane excitability in the early development of rat spinal cord. Our technique incorporated a membrane potential–sensitive fluorescent dye in conjunction with digital video microscopy to record the responses from over 3200 cells acutely dissociated from different

Received June 25, 1992; revised Oct. 2, 1992; accepted Nov. 18, 1992.

We thank G. D. Lange for helpful discussions relating to statistical analysis and formulating hypothetical distributions, and D. G. Schoenberg for assistance in editing the manuscript.

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ages and regions of the embryonic rat spinal cord. Each cell was monitored individually so that responses to several ligands presented successively could be evaluated for each. Our results indicate that, by the late embryonic period, the majority of cells developed sodium channels and receptor-activated $GABA_A$ and kainate-activated channels. As embryonic development progressed, functional sodium channels appeared prior to $GABA_A$ receptors, which were expressed prior to the kainic acid type of glutamate receptor. This pattern was present in all regions of the spinal cord, with developmental delays that reflected neurogenic gradients. The pattern of development on individual cells was highly stereotyped, with responses emerging in a sequential manner on the individual cell level to produce the pattern seen in the overall population.

Some of these results have previously appeared in preliminary form (Walton et al., 1990).

Materials and Methods

Cell preparation. Timed-pregnant Sprague–Dawley rats were killed with CO_2. Embryos were removed from the uteri by cesarean and placed into phosphate-buffered saline at room temperature. Embryonic age was confirmed by measuring crown–rump length (Hebel and Stromberg, 1986) of the embryos. Spinal cords were removed, and either the cervical or lumbar region was divided into dorsal and ventral halves for separate dissociation. At the ages examined, the dorsal commissure of the spinal cord has not completely fused and the spinal cord was readily opened along the commissure. Dorsal portions were then lateralmost in the splayed open preparation and a difference in translucency was apparent between the dorsal and ventral regions. The two dorsal regions were cut away from the ventral portion, using the translucency as a visual guide. Because of the known ventral-to-dorsal and rostral-to-caudal gradients of neurogenesis that occur in the rat spinal cord (Nornes and Das, 1974; Altman and Bayer, 1984), these distinctions in regions were made to narrow the spread in developmental stage of the cells examined within any single recording. Cervical cells were utilized for the initial series of experiments because they are the first to proceed through terminal mitosis within the spinal cord. Excised spinal cord portions were placed into a solution containing 20 U/ml papain (Worthington Biochemical, Freehold, NJ), 0.005% DNase (Boehringer Mannheim), 0.5 mM EDTA, and 1 mM L-cysteine for 45 min at 37°C (Huettner and Baughman, 1986) to initiate dissociation of the cells. After trituration to disperse the cells as completely as possible, the cells were spun at 300 × *g* for 5 min, and then resuspended in Earl's Balanced Salt Solution (EBSS) with 1 mg/ml bovine serum albumin (BSA; Sigma) and 1 mg/ml ovomucoid trypsin inhibitor (Sigma). The cell suspension was layered over 5 ml of EBSS with 10 mg/ml each of BSA and ovomucoid and centrifuged at 80 × *g* for 7 min. Cells were then resuspended in extracellular medium (ECM) containing (in mM) 140 NaCl, 5 KCl, 1 $MgCl_2$, 1.5 $CaCl_2$, 10 HEPES, and 6 glucose; pH 7.4, osmolarity 325 mOsm (adjusted with sucrose). A 0.25 ml aliquot of cell suspension containing 350,000–450,000 cells was placed in a central spot within a 35 mm^2 plastic culture dish that had been previously coated with 53 kDa poly-D-lysine (5 μg/ml; Sigma). The dishes were placed into a humidified incubator at 37°C where they remained until used for recording and for at least 1 hr to allow cells to adhere. All recordings were carried out on cells that had been dissociated in the morning of the same day. Recordings were obtained within a 3–5 hr period from several dishes, using only one field per culture dish.

Fluorescence measurements. A dish of cells was placed on the stage of an inverted microscope (Diaphot model, Nikon) equipped with a xenon arc lamp arranged for epi-illumination. An image intensifier (KS-1380, Opelco, Washington, D.C.) coupled to a CCD video camera (model 72, Dage) was attached to the video output port of the microscope. The dish was perfused continuously with flowing ECM at approximately 1.2 ml/min. Two glass inserts were used to decrease the dish volume to approximately 0.2 ml, so that the medium covering the cells was exchanged approximately six times per minute. The membrane potential-sensitive oxonol dye $DiBaC_4(5)$ (50 nM; Molecular Probes, Eugene, OR) was added to the superfusion media to maintain a constant extracellular dye concentration. The cells and all media-exposed surfaces were allowed to equilibrate with the oxonol dye for 15–20 min prior to beginning measurements. The microscope was equipped with filters appropriate for epifluorescence observation of the dye emission (excitation, 540 ± 15 nm; dichroic, 565 nm; emission, 580 nm high pass). A suitable field of cells was selected for recording, and a phase image (Fig. 1*A*; 20× objective) was captured into a digital video imaging work station built using a framegrabber board (PIP-1024, Matrox, Dorval, Quebec, Canada) installed in a 386-based computer. Each cell in the field was individually outlined on the phase image (Fig. 1*B*) along with a nearby area devoid of cells for background fluorescence measurement, and the outlines were stored as a cell map within the computer. Fluorescence images (Fig. 1*C*) were acquired at the rate of one per minute and either stored for later analysis or immediately analyzed with the previously made cell map. Cells were recorded initially for at least 6 min under baseline conditions before testing the various ligands or alterations in the superfusing ECM. Veratridine (25 μM) was used to probe for the voltage-gated sodium channel. Muscimol (2.5 μM) was used for activation of the $GABA_A$ receptor–linked channel, and kainic acid (50 μM) for the non-NMDA glutamate receptor–activated channel. Preliminary experiments showed that these concentrations were saturating and elicited the greatest response. Every field of cells was exposed to each of the three ligands, and only one field was examined in each culture dish. A cell's response to a ligand did not depend upon the order of ligand exposure (see Fig. 3*B*). Because a minority (10–20%) of cells would exhibit prolonged recovery after the multiple-minute exposure to kainic acid, this ligand was applied third in the sequence. Test doses were applied for either 3 or 6 min with periods of 7–12 min of physiological ECM superfusion between test doses. No differences in analyzed results were seen between experiments with 3 or 6 min exposure to ligands. 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX) was obtained from Tocris Neuramin (Bristol, UK). All other chemicals and ligands were obtained from Sigma Chemical Co. (St. Louis, MO).

Analysis consisted of summing the total fluorescence within the outlined area defined for the cell, subtracting the fluorescence of an equivalent area of the nearby background, and storing the background-subtracted cellular fluorescence. This yielded a sequence of fluorescence values in time for each individual cell. The anionic oxonol dyes achieve membrane potential sensitivity by diffusing across the cell membrane to arrive at a Nernstian equilibrium (Apell and Bersch, 1987). They indicate depolarizing changes by arriving at a higher cellular concentration than at more hyperpolarized potentials. Depolarization is thus recorded as an increase in fluorescence (Fig. 2*B*). Because the distribution of the dye should follow a Nernstian relationship to membrane potential, the logarithm of cellular fluorescence is expected to be linearly related to membrane potential. This has been demonstrated for a different dye, of the cyanine family (Plasek and Hrouda, 1991), and thus all measurements were performed using the logarithm of fluorescence normalized to the mean fluorescence during the initial control period, and are presented as changes in log(fluorescence). This method does not allow attributing an explicit membrane potential with any cell's particular level of fluorescence, but does enable changes in membrane potential to be clearly detected.

Analysis continued by plotting each cell's data on the computer screen, and indicating the amplitude of response manually with an on-screen cursor. Each cell's fluorescence values over time were thus reduced to a list of the amplitude of change in the cell's normalized log fluorescence for each condition presented. Initial investigations showed that the intrinsic variability in fluorescence intensity of unstimulated cells was generally less then 0.1 log units. This was taken as the threshold for considering a cell's change in fluorescence to be a response. Cells not showing adequate recovery during the wash period were discarded. Some cells showed extremely high fluorescence relative to the majority, and this minority (generally 2–10%) of cells were not utilized in analysis. The very high fluorescence of these cells likely indicates a relatively unpolarized state. These cells may have been unhealthy or depolarized for other, unknown reasons. To calculate mean results, the cells in each field (generally 30–50) were combined to produce a percentage responding to each experimental condition. These values were then averaged with the corresponding percentages for other fields of cells from the same age and spinal cord region to provide mean and SEM percentage responses. Over 3200 cells are reported in this study of ligand responses. The experiments reported here utilized 84 different fields of cells from 34 different dissociations. Specific numbers of fields for each age and region are given in Table 1 and the figure caption that first presents the numeric data. Additional fields and dissociations were utilized in preliminary experiments to develop the specific procedures and demon-

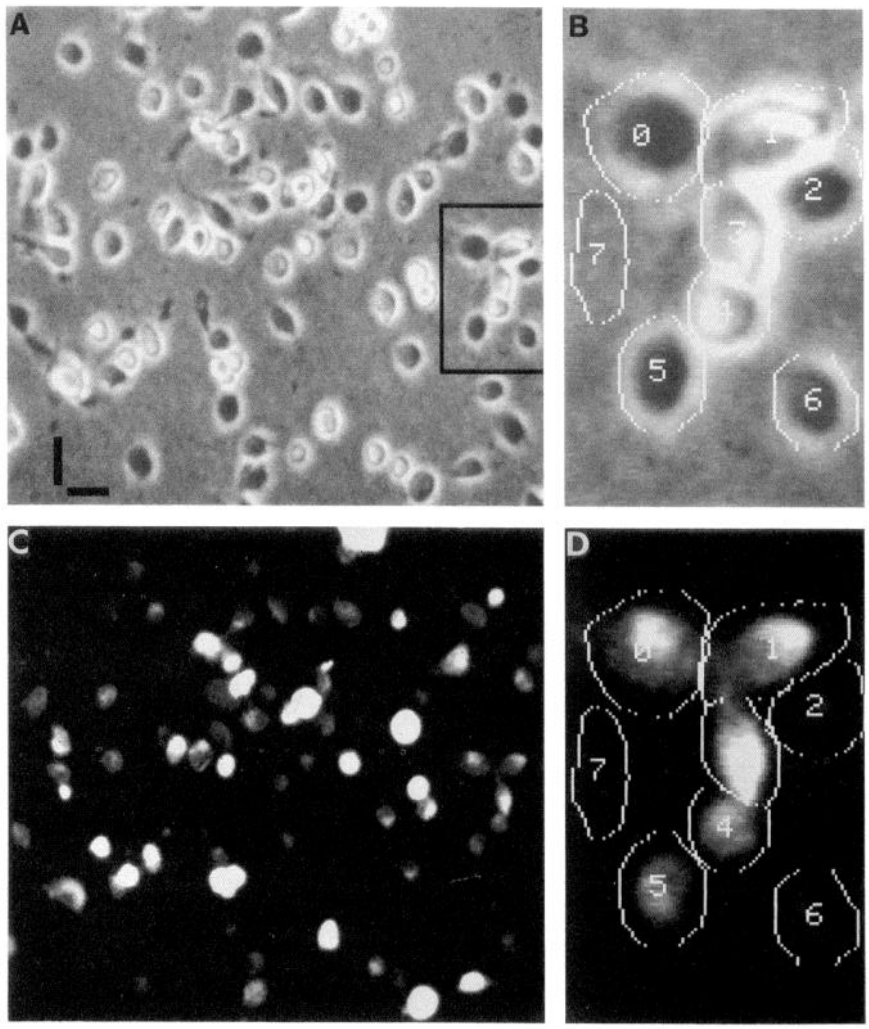


Figure 1. Video appearance of dissociated cells. The cells were dissociated from E17 cervical ventral spinal cord and allowed to adhere for approximately 1 hr. *A*, Phase-contrast appearance of the cells through a 20× objective (scale bars are both 20 μm). The cells are well dissociated and adhered as a monolayer. Some of the cells have started to redevelop processes. *B*, An enlarged view (3×) of the marked portion of *A* illustrates that the cells are easily visually separable for encircling. "Areas of interest" *1–6* encircle cells for subsequent measurement of fluorescence; area *7* is a nearby portion without cells for background fluorescence measurements. Although some cells are adjacent, they are distinguishable in the enlarged view used for creating the marked areas. *C*, The same field of cells after equilibration with oxonol and viewed in fluorescence, with intensities adjusted to optimize measuring fluorescence over a wide dynamic range of levels. *D*, The same portion of the field as in *B*, with the cellular fluorescence within the marked areas. The small difference in horizontal and vertical scale size (*bars* in *A*) is due to slight differences in the video camera, image capture board, and image printing scaling of individual image pixels.

strate reproducibility of responses, specificity of responses, and so on. Example data from these experiments are utilized in Figures 2*B* and 3. Results obtained with cells of same age and location but different dissociations were largely consistent, as indicated by size of error bars in figures showing averaged data.

Statistical analyses were performed using computer software (SIGMAPLOT, Jandel Scientific, San Rafael, CA; CSS:STATISTICA, StatSoft, Tulsa, OK). Statistical significance was defined by the criterion of $p < 0.05$ and all statements of significance refer to this criterion.

Results

Cell fluorescence changes in response to specific channel-opening agents

The appearance of a typical video-captured field of cells is shown in Figure 1*A* in phase-contrast technique; the oxonol fluorescence of the same field under baseline conditions is seen in Figure 1*C*. The individual cells were easily recognizable and attached as a monolayer. Although cells frequently adhered to the culture dish adjacent to one or more other cells, they could be visually separated into different "areas of interest" for separate measurements of each cell's fluorescence (Fig. 1*B*). During the course of a typical 1–2 hr recording, the cells remained largely in the same position within the video field. A small number of cells did move position, generally less than one cell diameter in distance. This occurred more frequently in the cells from younger embryos, but was not systematically evaluated. Most movements were directed such that the cell body moved toward or away from that of another cell that it had been in close proximity to and had contacted with a thin process. The rare cell that could not be readily enclosed by a single outlined area of interest due to excessive movement was discarded. The absolute fluorescence of the individual cells varied within the field, most likely relating to differences in cell size and dye binding to cellular components (Brauner et al., 1984) as well as differences in resting membrane potential.

In this study we focused on three forms of membrane excitability: the voltage-activated sodium channel, the $GABA_A$ receptor–coupled channel, and the kainic acid–activated type of non-NMDA glutamate channel. Veratridine was used to probe for the sodium conductance. Muscimol was used to activate the $GABA_A$ receptor, and kainic acid for the non-NMDA glutamate receptor. Depolarizing responses seen with these agents were clear (Fig. 2*A*) and reproducible (Fig. 3*A*). There were depolarizing responses for all the agents. While this is expected for the sodium channel and kainate-activated channel openings, the response to muscimol was also depolarizing. This is somewhat different from what might be expected for a transmitter that plays an inhibitory role in the mature nervous system. Occasionally, hyperpolarizations were also recorded in response to

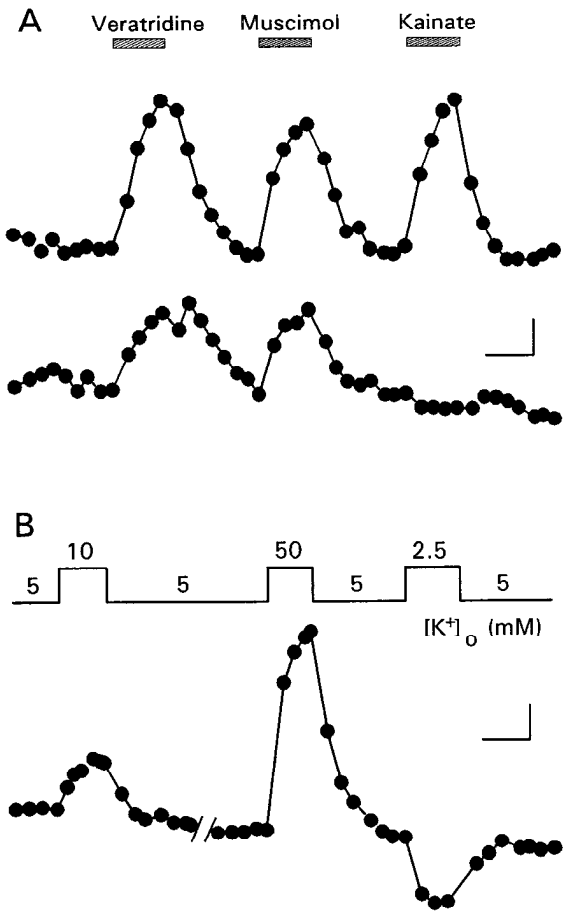
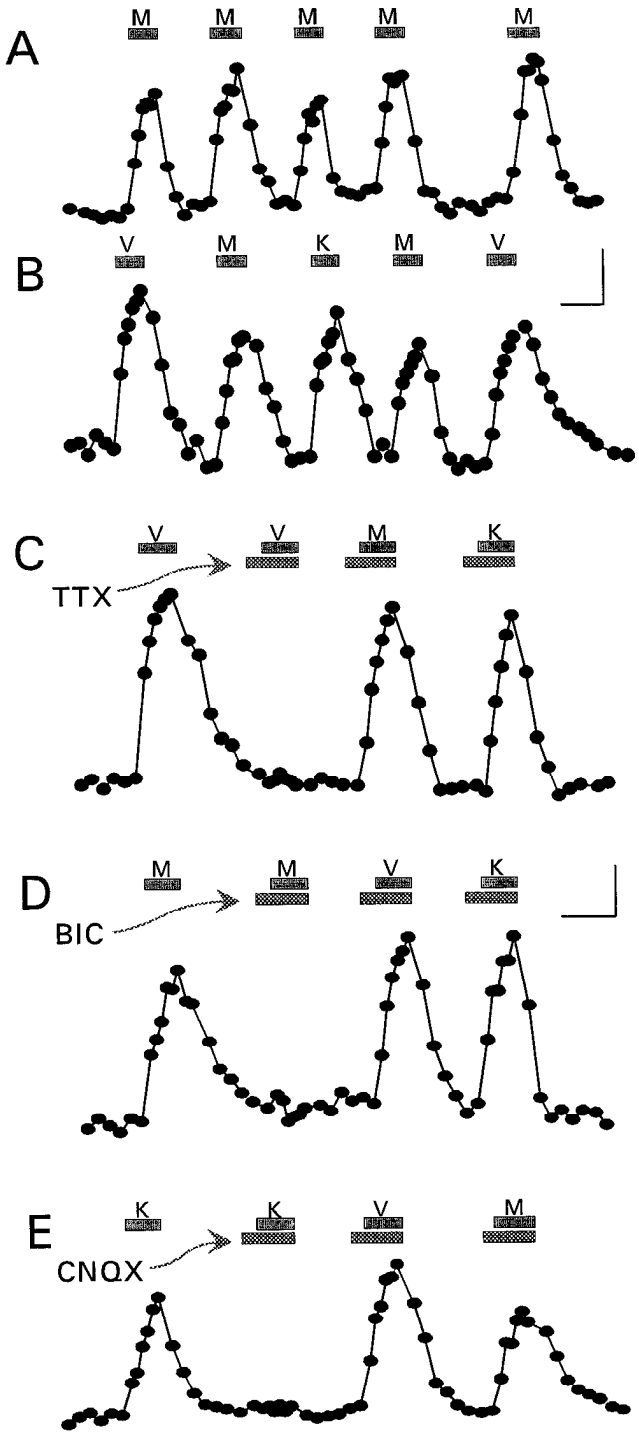


Figure 2. Fluorescence changes of individual cells are clearly resolvable. *A,* Measurements obtained from two individual E15 cervical dorsal cells successively exposed to the three ligands (indicated by *hatched bars*). One cell shows responses to all three ligands (*upper trace*), while another cell has responses only to veratridine and muscimol (*lower trace*). The initial baseline period is stable, and good recovery is seen on washout of ligands. *B,* Fluorescence responses of an E17 cervical dorsal cell with changes in external $[K^+]$ show both rises in fluorescence (reflecting depolarization) with raising of external $[K^+]$ and decreases (reflecting hyperpolarization) with a lowering of $[K^+]$. External $[K^+]$ was 5 mM except when altered by equimolar replacement of sodium by potassium as indicated along the concentration indicator line. Calibration: vertical, 0.1 units log fluorescence in *A* and *B*; horizontal, 5 min in *A*, 6 min in *B*.

each of these tested ligands. However, they were typically small in amplitude and not reproducible with repeated exposure, in contrast to the depolarizing responses, which were quite reliable. They are not considered further. The specificity of the responses was investigated pharmacologically using classical antagonists. TTX (1 μM) blocked the response to veratridine (Fig. 3*C*), bicuculline methobromide (50 μM) eliminated the response to muscimol (Fig. 3*D*), and CNQX (20 μM) prevented the response to kainate (Fig. 3*E*). Sodium-free ECM (*N*-methyl-D-glucamine replacement for sodium, 140 mM) abolished responses to both veratridine and kainate, but not to muscimol (not shown). These results indicate that the responses observed to the test ligands



application does not affect a cell's ability to respond to subsequent ligand exposures. An E17 cell exposed to all three ligands shows a similar response to a repeat exposure of a ligand, and appears largely unaffected by the ligand exposures that occur prior to any particular exposure. *C,* TTX (1 μM; *row of bars below ligand markers*) specifically blocks the response of sodium channels opened by veratridine, but does not block the response to muscimol or kainate. *D,* Bicuculline methobromide (*BIC*; 50 μM) specifically blocks the response to muscimol, but not to veratridine or kainate. *E,* CNQX (20 μM) blocks the response to kainate, but not to veratridine or muscimol. Ligand application indicated by *marker bars* positioned above respective fluorescence recording: *M,* 2 μM muscimol; *K,* 50 μM kainate; *V,* 25 μM veratridine. In *C–E* blocker application is indicated by marker bar located just below ligand marker bars. Calibration: vertical, 0.1 unit log fluorescence; horizontal, 5 min. Upper calibration applies to *A* and *B*; lower calibration applies to *C–E*.

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Figure 3. Individual cellular responses to ligands are reproducible and specific. *A,* A cell repeatedly exposed to 2 μM muscimol shows a reproducible response during five sequential exposures. *B,* The order of ligand

Table 1. Number of cells occurring within each response category by age and region

Age and region	Response category								Total cells	*n*
	- - -	V- -	VM -	VMK	- M -	- MK	V- K	- - K		
Cervical										
E13 Ventral	126	37	23	10	9	2	4	4	215	6
Dorsal	84	46	11	7	3	1	7	4	163	4
E15 Ventral	26	13	27	149	12	13	8	3	251	7
Dorsal	53	48	52	121	16	5	12	2	309	7
E17 Ventral	14	2	3	95	2	14	21	0	151	5
Dorsal	6	2	6	145	4	18	12	2	195	6
E19 Ventral	8	1	0	81	1	13	14	6	124	5
Dorsal	17	0	1	101	1	23	27	4	174	6
E21 Ventral	24	1	1	151	2	24	10	4	217	6
Dorsal	13	2	0	126	0	12	27	3	183	5
Lumbosacral										
E15 Ventral	136	47	101	215	39	32	22	5	597	14
Dorsal	256	107	111	120	40	16	34	18	702	13

Response category naming is as in Figure 8. Number of cells is sum over all fields for each age and region. *n* indicates number of fields.

were due to specific activation of sodium conductance, $GABA_A$ receptors, and kainate-activated glutamate receptors.

Responses develop sequentially in the embryonic cervical spinal cord

For each type of excitability examined, progressively larger percentages of cells responded as embryonic age increased (Fig. 4). At embryonic day 13 (E13), the earliest age examined, responses to veratridine were already present on 37–43% of the cells, increasing to 75–78% at E15, and responding percentage plateaued at this level for the rest of the embryonic period. The percentage of veratridine-responding cells from both dorsal and ventral regions was not significantly different at each age studied. The percentage responding at E13 to muscimol (16–21%) was less than that recorded for veratridine. Responses again greatly increased at E15, to 79% for ventral cells and 63% for dorsal cells, and this was a statistically significant lead in the development of the response to muscimol for ventral over dorsal cells. By E17, similar percentages responded to muscimol in both dorsal and ventral regions.

Development of cellular responses to kainate paralleled those for muscimol. Starting from a low percentage (9–16%) for both ventral and dorsal cells at E13, the percentages responding increased by E15 with a significant lead in the development of ventrally derived cells (69%) over dorsal cells (45%). There was a further increase in percentage responding to kainate between E15 and E17 for both dorsal and ventral regions. For the response to muscimol, this increase in responding percentage from E15 to E17 had occurred only for dorsal cells.

There were statistically significant differences in the development of the different types of excitability (Fig. 5). Dorsal cells exhibited a significant appearance of veratridine responses prior to either muscimol or kainate responses at E13. The lead in sodium channel appearance over the two chemical forms of excitability was maintained at E15, but largely equalized by E17, when the majority of cells responded to all three ligands. Dorsal percentages responding to muscimol and kainate were similar at E13, but by E15 significantly more cells exhibited responses to muscimol than to kainate. The kainate-responding percentage was again similar to the muscimol-responding percentage by E17.

In the ventral portion of the cervical spinal cord, the pattern of response development was similar to that of the dorsally derived cells, but was expressed earlier in embryonic age. Veratridine-induced responses were present on a statistically significant greater percentage of cells then either muscimol or kainate responses at E13. In addition, significantly more cells responded to muscimol at E13 than to kainate. By E15 the muscimol-responding percentage was equivalent to that for cells responding to veratridine, and both were significantly greater than the kainate-responding percentage.

These results indicate that the three forms of membrane excitability studied in the embryonic cervical spinal cord develop early, and rapidly become widespread throughout the majority of cells. Their expression in the population occurs sequentially, with sodium conductance appearing prior to $GABA_A$ receptors, which in turn emerge before kainate-activated receptors. These responses also show a ventral before dorsal gradient of development.

Cellular excitability emerges along a rostrocaudal gradient

To examine further the sequence of development as well as adding rostrocaudal comparisons, cells dissociated from lumbosacral portions of E15 spinal cord were studied. The results obtained with E15 lumbosacral cells (Fig. 6*A*) reveal a statistically significant lead in the expression of excitability in ventrally derived cells over dorsally derived cells for all three responses. Also readily apparent was the developmental lead in expression of excitability in the cervical region relative to the lumbosacral region for all three responses. Interestingly, for the responses to muscimol and kainate, the ventrodorsal difference in percentage responding was similar in size to the rostrocaudal difference at E15.

The lumbosacral results, when examined for sequence of channel appearance (Fig. 6*B*), recapitulated the sequence seen in cervical cells. In dorsal cells, responses to veratridine were significantly more numerous than those to muscimol, which were in turn significantly more widespread than those to kainate.

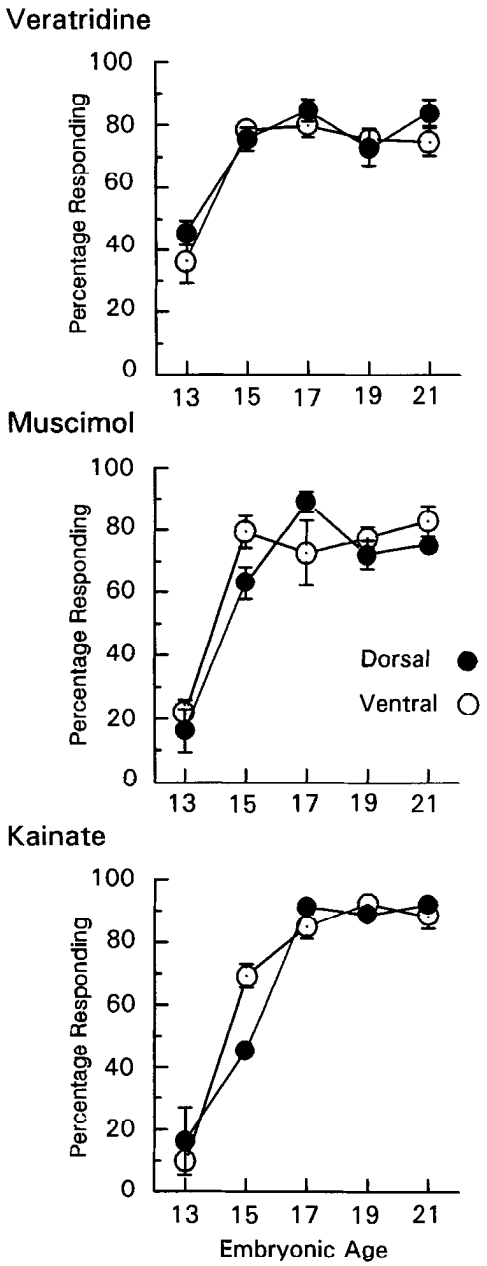

Figure 4. Cellular responses to each ligand become progressively more widespread in the population over the earlier embryonic ages. Mean percentages ± SEM of responding cells in cervical spinal cord from E13 to E21 in dorsal and ventral regions are shown in each graph. The percentage of responding cells increases for each ligand from its value at E13 to a plateau level reached by E17 for all three ligands. Differences between dorsal and ventral development of responses can be seen at E15 for both muscimol and kainate. Error bars not apparent on some of the symbols, especially in kainate responses, are of a height equal to or less than the symbol size. Number of fields examined at E13, E15, E17, E19, and E21, respectively: dorsal 4, 7, 6, 6, 5; ventral 6, 7, 5, 5, 6.

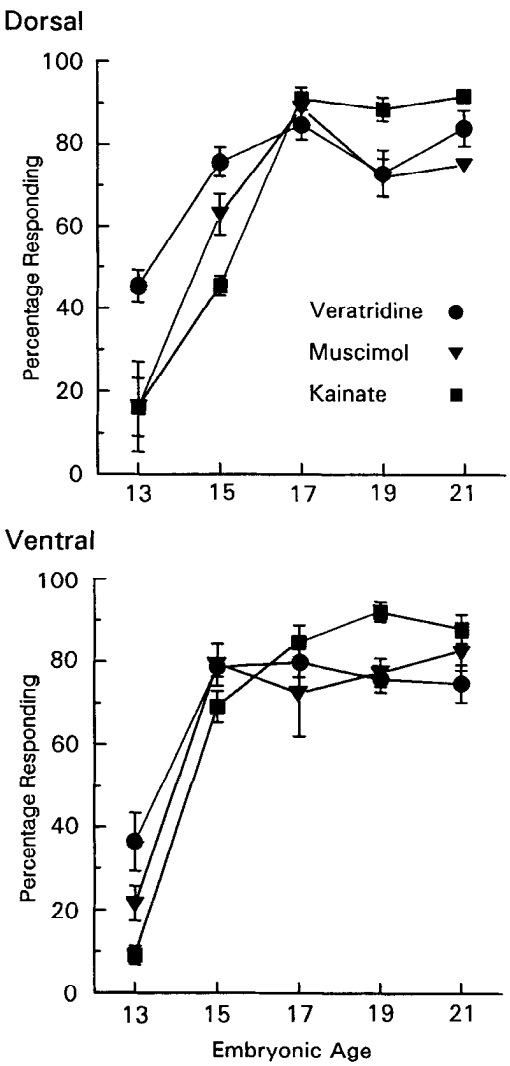

Figure 5. The course of development of response is different for each ligand. The mean ± SEM percentages are shown to compare responses to the different ligands within each half of the cervical spinal cord. For both the dorsal and ventral data, veratridine responses appear on a larger percentage of cells than muscimol responses, and muscimol on a larger percentage than kainate at one or more ages. The clearest differences between the three ligand responses appear at E13 in ventral cells and at E15 in dorsal cells.

For ventral lumbosacral cells, veratridine- and muscimol-responding percentages were similar, and both were significantly greater than those to kainate. This is the same pattern as was seen in cervical cells. These results further support the conclusion that these membrane properties become expressed along a ventral-to-dorsal gradient, as well as with a sequence of emergence of channels as had been seen in cervical cells.

These experiments each illustrate a moment in the developmental sequence of events. Because there are clearly large changes occurring between some of the 2-d-separated cervical measurements obtained, we wished to investigate if there were significant changes in the cellular responses over the course of the several hours following dissociation. To examine this, a larger number of fields were measured for responses in the E15 lumbosacral experiments than had been utilized in the cervical studies. This number was greater in number of fields utilized from each dissociation (generally four fields for lumbosacral, two or three fields for cervical) as well as in total fields (13 lumbosacral dorsal fields, 14 ventral fields). Fields of cells were examined beginning 1 hr after dissociation, and evenly spaced so that the fourth field was completed approximately 6 hr after dissociation. Log-linear analysis of the four-way contingency table (sequence order of field × veratridine responding × muscimol responding × kainate responding) was utilized to look for interactions between

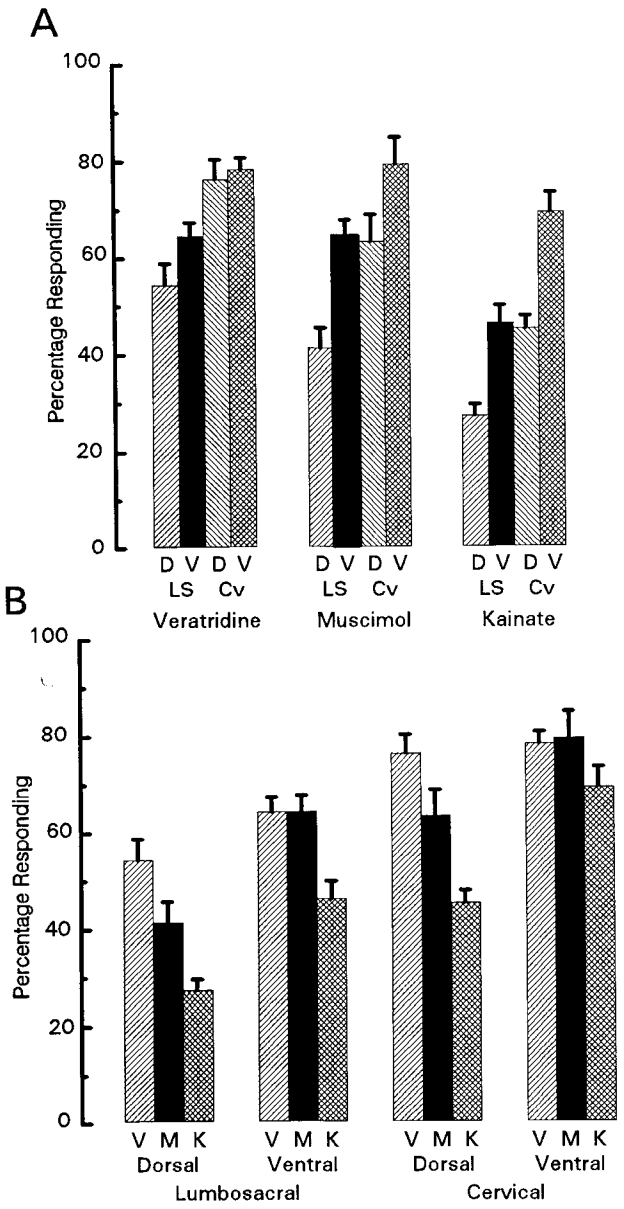

Figure 6. E15 lumbosacral cells show ventrodorsal gradients similar to cervical cells as well as rostrocaudal gradients. Mean ± SEM percentages of E15 cells that responded to each ligand are compared for the different regions studied. *A*, Comparison of regional differences for each ligand. In the lumbosacral regions, as in the cervical, dorsal cells responded less frequently than ventral cells. Lumbosacral response percentages were also less than their cervical counterparts. *D*, dorsal cells; *V*, ventral cells; *LS*, lumbosacral cells; *Cv*, cervical cells. *B*, Comparison of ligand differences within each region. E15 lumbosacral cells show a pattern of ligand response similar to that seen in cervical cells. In dorsal cells, veratridine (*V*) percentage was higher than muscimol (*M*) percentage, which was higher than kainate (*K*) responding percentage. The gradient of veratridine greater than muscimol percentages responding was already equalized at E15 in ventral cells from both lumbosacral and cervical regions. Number of fields: cervical, 7 both dorsal and ventral; lumbosacral, 13 dorsal and 14 ventral.

the field sequence within this 6 hr period and the expression of responses (data not shown). No significant interactions were found for either the ventral cells or the dorsal cells between recording sequence during the afternoon and responses expressed by the cells. This indicates that there was no clear progression in expression of responses during the time the cells were held in dishes awaiting recording.

Excitability develops sequentially at the single-cell level

Thus far, we have examined the appearance of specific forms of excitability in entire populations of spinal cord cells from specific regions and demonstrated the sequential appearance of these responses, but have not directly addressed the question of developmental sequence at the individual cell level. The data analysis provides an amplitude of response measurement for each of the three ligands for every individual cell. This conceptually allows three-response histograms to be formulated for each region at each age. Taking only two of the ligand responses at a time allows viewing the data as a two-dimensional density plot. Figure 7 shows, for all cells, the kainate response versus the veratridine response of each cell. At E13, the cells responded variably to veratridine but little at all to kainate. Hence, many cells exhibited functional sodium conductance without functional kainate-activated conductance. In the oldest ages, a majority of cells had a response to both ligands, and showed a correspondence of response amplitude between veratridine and kainate responses. The middle ages, especially E15, were the most complicated in distribution. Most cells had developed functional sodium conductance and were in the process of developing responses to kainate. Noteworthy was the fact that there was no high-density cluster of cells that showed a response to kainate without one to veratridine. This indicated that the great majority of cells would, as individuals, develop sodium conductance prior to kainate-activated conductance, conforming to the sequence that had been seen in the population overall. Both other two-response density plots (veratridine vs muscimol, muscimol vs kainate) provided views (not shown) that also paralleled the sequence seen in the region overall. Individual cells expressed veratridine responses before muscimol responses, and muscimol responses before kainate. There were no high-density clusters of cells showing a muscimol response without veratridine or a kainate response without muscimol.

Because of their complexity, these plots are difficult to interpret fully. In order to delineate better the patterns inherent in the data, the responses for each cell have been used to classify each cell into one of eight groups, depending on the presence or absence of a response to each of the three ligands. The results of this classification for E13 cervical ventral cells are shown in Figure 8. The leftmost group of four elements represents the individual cells that are consistent with the sequential development of channels in the order of sodium channel before $GABA_A$ activated before kainate activated. The columns in Figure 8 are ordered in the pattern of earliest (no response to any of the three ligands) to latest in the sequence (responses to all three ligands). The rightmost group of four elements represents the cells that appear to have broken the sequential pattern, displaying a response to one of the ligands without a ligand response that should have developed earlier. In E13 ventral cervical cells, the largest group is cells showing no response to any ligand, with a progressive decrease in percentage of cells in the later stages of the development sequence. There are some cells in the pattern-breaking groups, but these constitute small percentages.

The data for all cervical cells studied were similarly categorized and averaged across fields (Fig. 9) or cell counts directly summed across fields (Table 1). These show that at E13, the largest single group comprises cells with no response to any ligand, while at the oldest ages, the largest group comprises those having all three channels. At E15, there is a distribution of cells in several groups; the great majority are within the groups con-

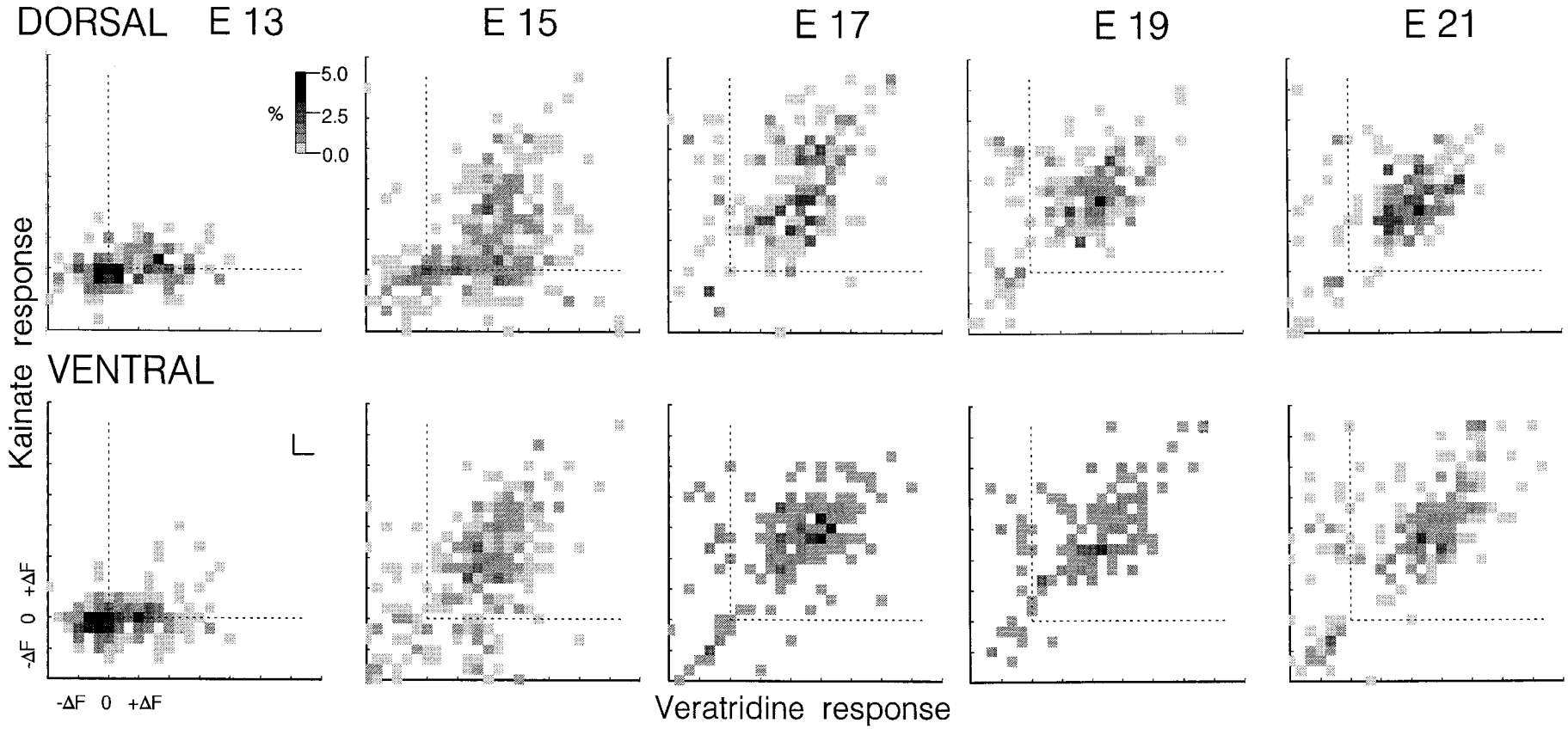

Figure 7. A two-response density histogram reveals a complex course of events in response development. The amplitude of response (change in log fluorescence) to veratridine and kainate is indicated by position along each axis. *Broken lines* within each graph indicate the zero change in fluorescence line. Each small *density block* indicates the percentage of cells that exhibited response amplitudes within the amplitude range covered by the block's position. Greater percentages are indicated by *darker density*. The data from all cervical cells examined are shown. This two-dimensional density histogram is a collapsed version (eliminating muscimol response differences) of the full three-dimensional density histogram that indicates the complete behavior of every cell studied. The patterns seen in prior figures are recognizable. At E13 many but not all cells respond to veratridine (broad spread of densities along *horizontal dimension*) while few of these cells respond to kainate (density clustering around the *horizontal broken line* marking zero change in fluorescence in the vertical, kainate response scale). At E15 many dorsal cells still do not exhibit depolarizing kainate responses, but now many cells that do reveal veratridine responses also show a kainate response (*vertically rising mass of densities*). Only a rare cell shows a kainate response without a veratridine response (*vertical broken line* marking zero veratridine response with only few adjacent densities). At later ages the majority of cells are clustered at *midgraph*, indicating responsiveness to both veratridine and muscimol. *Density scale* indicates percentage of all cells in each age and region that exhibit the appropriate response amplitudes (as indicated by the *density box* location), shown as darker density for higher percentage. *Amplitude scale* (at *upper right* of *E13 ventral cervical graph*) indicates 0.1 units of change in log fluorescence.

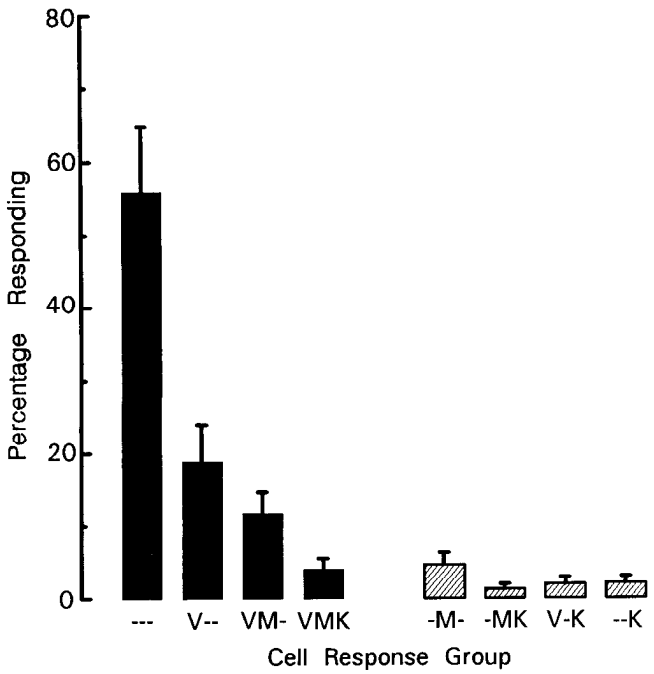

Figure 8. Categorized response groups simplify interpretation of the distribution of cellular responses. E13 cervical ventral cells were divided into the eight possible response categories depending on whether or not they responded to each ligand. The response category names indicate presence of a response to veratridine (*V*), muscimol (*M*), or kainate (*K*) by containing the corresponding letter, no response by having a *dash* in the corresponding location. *V*- - indicates cells that had response to only veratridine; - *MK*, cells that had response only to muscimol and kainate; and so on. Categories are arranged so that the left four groups (*solid bars*) are cells consistent with the progression of veratridine response appearing before muscimol response before kainate response. The right four groups (*hatched bars*) are cells that are not consistent with this sequential progression. This figure shows that the majority of cells occur within the categories consistent with sequential appearance of responses. Additionally, at E13, most are in the earlier stages of the sequence. Few cells exhibited a set of responses that placed them in the rightmost group of four categories. This same pattern of categories and labeling is maintained throughout the following figures.

sistent with the sequential development of channels on each cell. At the earliest two ages, when most channel development is occurring, the pattern-breaking groups are all very small. In the later ages these groups show small but nonzero percentages of cells. These are mostly cells that show the presence of two of the forms of excitability but lack responses to either veratridine or muscimol.

The data obtained on lumbosacral-derived cells may be grouped similarly (Fig. 10). This again shows the great majority of cells in the groups consistent with sequential development of channels in both the dorsal and ventral portions of the lumbosacral spinal cord. Only a very small percentage of cells are in any of the sequential pattern-breaking groups. This demonstrates that differences in development between the ventral and dorsal portions of spinal cord occur caudally as they did rostrally. Also clearly reinforced is the rostrocaudal gradient in development, with the E15 lumbosacral pattern of groups appearing as a less mature distribution than is seen in the cervical E15 cells. Thus, the E15 lumbosacral data are similar to what might be expected of cervical data from an age intermediate between E13 and E15.

These data suggest that development of responses is occurring in an ordered sequence on at least the majority of cells. In order to test this further, we posited the two extremes of developmental patterns and tested them against the observed patterns. The first proposition tested was that all cells develop each response independent of those that had already developed. To test this hypothesis, we used the overall percentage of cells for each age and location to generate a hypothetically expected distribution of responses among the eight groups of response combinations (e.g., Fig. 11), and calculated a goodness-of-fit χ^2 statistic to compare the observed and postulated distributions. This was performed for each of the 12 different age and region categories (Fig. 12). In all but one case, the postulated independent development distributions were statistically significantly different from the observed, allowing rejection of this postulated pattern of development. The one case that did not reject the postulate was that of the E17 cervical dorsal region.

The second extreme proposition tested was that all cells showed strictly ordered development: sodium channels before $GABA_A$ receptors before kainate-activated receptors. This implies that any cell showing a response pattern violating this sequence was, in effect, an erroneously categorized cell. To test this postulate, the responses within each age and region category were reviewed and a hypothetical distribution formulated by shifting any cells that fell within one of the four now "prohibited" response groups into one of the four allowed groups by changing only one of its responses. The - M - responses were split into the VM - and --- (no-response) groups, the - MK responses placed into the VMK group, and similarly for the remaining two prohibited groups. This distribution was then used to calculate a χ^2 statistic for goodness of fit to the observed distribution. Of the 12 age and region categories, 8 did not support rejecting the postulated distribution. The E19 and E21 cervical dorsal, and both E15 lumbosacral sets of observed data were significantly different from the postulated distribution. Except for these four cases, the response distributions observed are consistent with the hypothesis that these forms of membrane excitability develop in a highly ordered, sequential manner on each cell individually.

Discussion

In recent years a wide variety of optical probes of cell physiology have become available. Numerous different dye probes of membrane potential have been used (Waggoner, 1979; Dasheiff, 1988). The specific dye used in the present study belongs to the family of oxonol dyes, which are well suited to investigating the plasma membrane potential in a variety of cell types (Bashford et al., 1985; Wilson and Chused, 1985; Apell and Bersch, 1987; Civitelli et al., 1987). The oxonol dyes are among the dyes that report membrane potential on a slow time scale. Slow-responding dyes generally function by diffusion through the cell membrane, reaching a concentration equilibrium that is dependent on the membrane potential, thus responding with a time course reflective of the diffusion process (Brauner et al., 1984; Clarke, 1991). While these dyes are not useful for investigating events on the millisecond time scale of action potentials or fast synaptic transmission, they have the advantages of low toxicity, usefulness in a wide range of cell types, and large percentage changes in fluorescence. However, this technique is only able to detect responses that cause a change in membrane potential. If a ligand opens a channel with an equilibrium potential at or very near the resting membrane potential, the fluorescent dye technique will be unable to detect it.

The technique of digital video fluorescence imaging used here

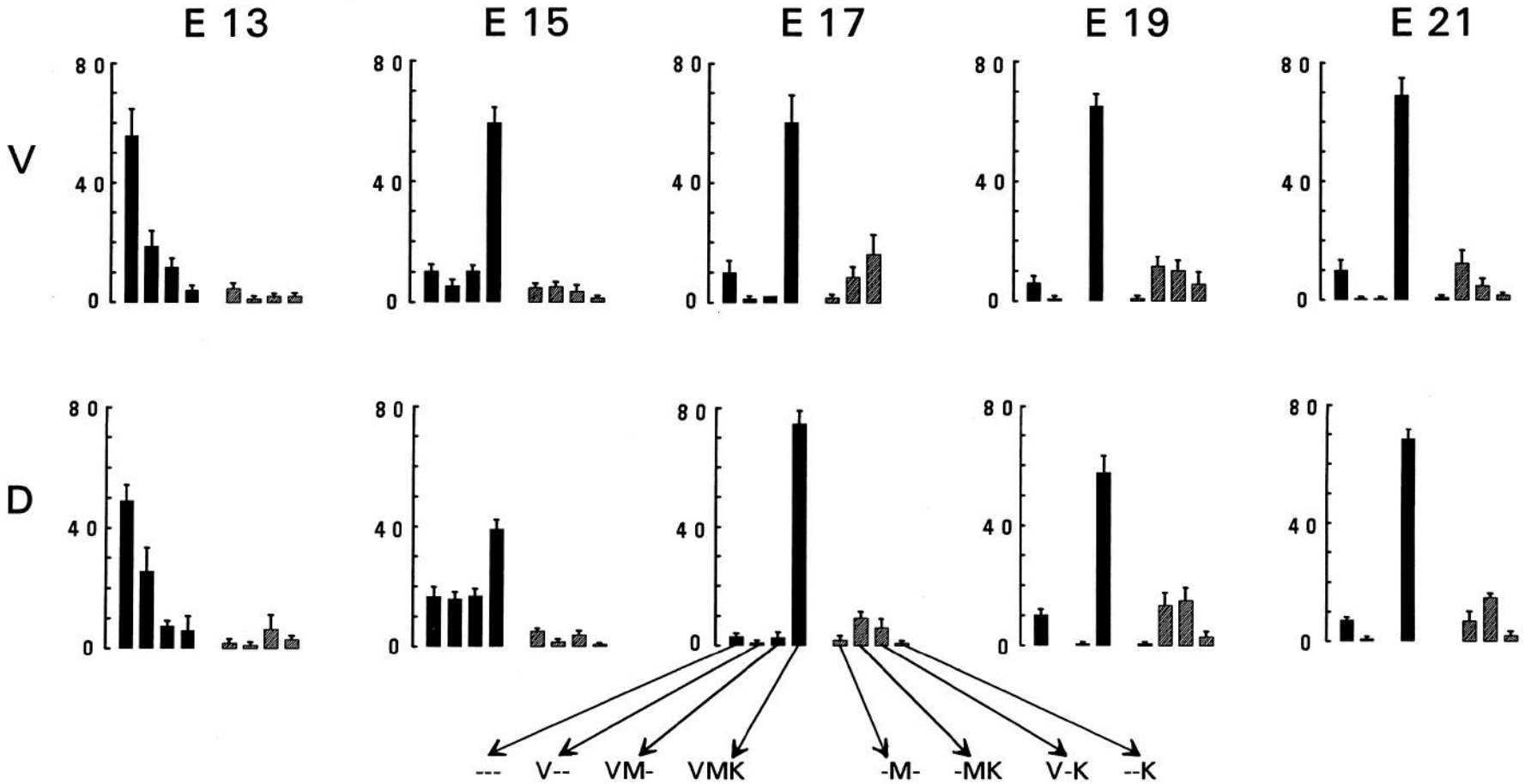

Figure 9. Most cells from the cervical region occur in the groups consistent with a sequential development of responses. All cervical cells were divided into the eight response categories (category pattern and labeling as in Fig. 8). Most cells are contained within the response groups consistent with sequential development of channels, although very noticeable percentages of cells fall into the nonconsistent groups at later ages, in both dorsal and ventral regions. At E19 and E21 there are percentages of cells with no responses to any ligand, possibly recently generated cells, but few cells are in the groups intermediate in the sequential progression (V-- and VM -) to showing all three responses (VMK). Instead, greater percentages appear in the non-sequential-consistent groups.

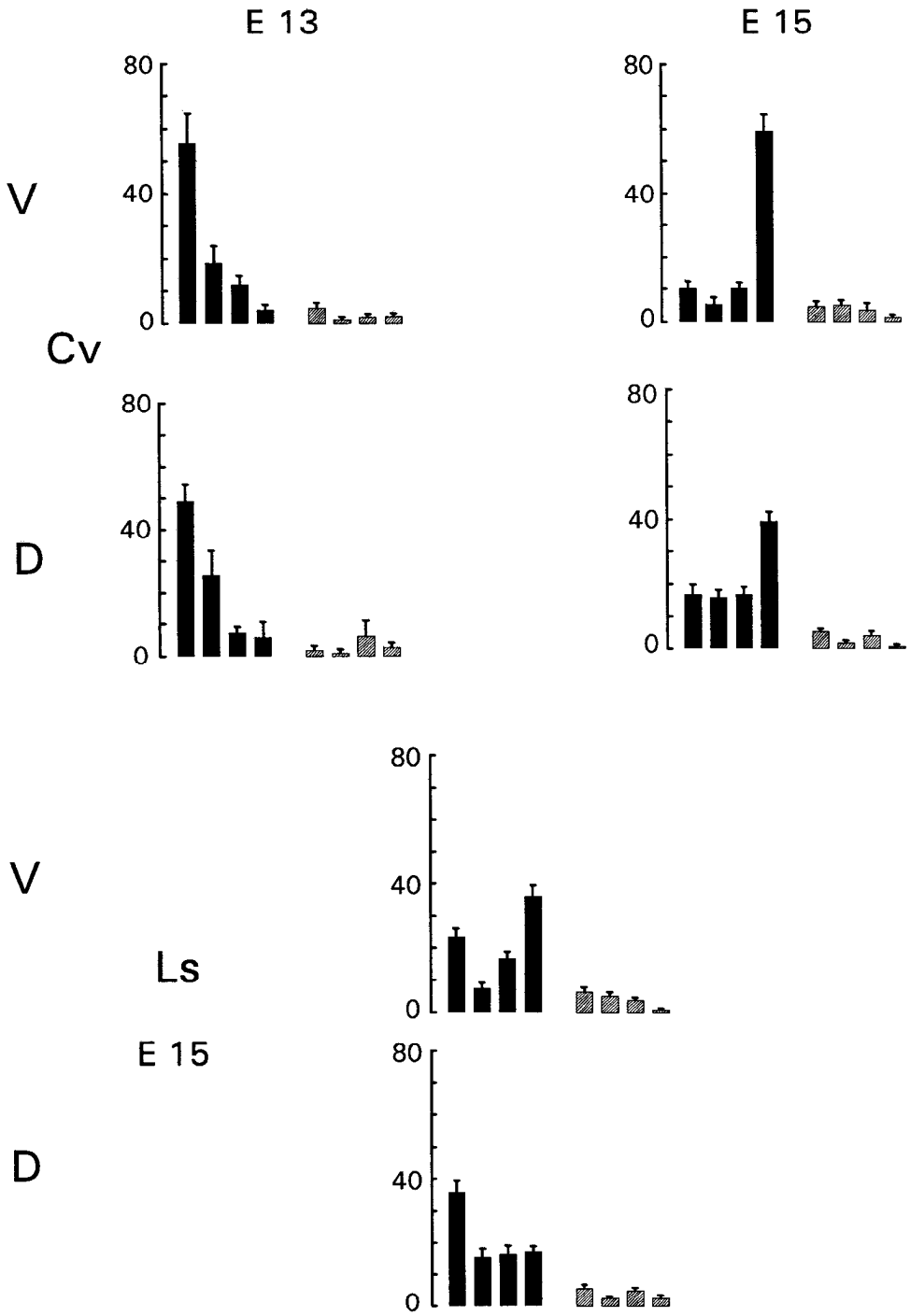

Figure 10. Lumbosacral regions also show most cells consistent with the sequential progression of channel appearance. Response category percentages for E15 lumbosacral cells and E13 and E15 cervical cells are shown (category patterns as in Fig. 8). The E15 lumbosacral cells show a pattern of distribution within the groups that appears less progressed than E15 cervical cells, but more so than E13 cervical cells. This figure also illustrates that the ventral pattern of response development is ahead of dorsal and that the cervical regions are more advanced than the lumbosacral counterpart.

is particularly well suited for addressing the issues we have investigated. In order to obtain a well-defined description of the behavior of the cells at each of the ages and regions utilized, it was necessary to measure a large number of cells so that the population was well sampled. The oxonol and related voltage-sensitive fluorescent dyes have been utilized for this purpose previously in flow cytometry systems (Wilson and Chused, 1985; Mandler et al., 1990). In flow cytometry experiments, however, each individual cell can be measured only once, and a separate population of cells is utilized for each control or test condition. This can give a qualitative picture of the way in which the population responds, but is not able to provide as clear a statement of the proportion of cells responding as can the present technique where each cell acts as its own control. Also importantly, the video microscopy technique's repeated measurements on each cell provides a specific set of responses for the multiple ligands, which characterizes each cell individually. In this regard, the video microscopy technique is more similar to microelectrode electrophysiology, where the response of the electrically recorded cell is continuously monitored. However, video microscopy offers a major advantage over microelectrode techniques for this type of study. Microelectrodes can record from only a single cell at a time, so a strong sense of the population of cells could be obtained only if vast amounts of time

were spent recording many cells, one at a time. Over 3900 cells were measured and analyzed during this study, a population size not feasible to study with microelectrodes.

Excitability emerges early in embryogenesis along characteristic gradients

The development of responses occurred over a short portion of the embryonic time period studied. In the cervical cells, responses to veratridine had plateaued by E15 in both dorsal and ventral regions, as had muscimol responses in the ventral portion of the spinal cord. Dorsal muscimol responses and kainate-activated responses had reached plateau levels by age E17. This is perhaps 2–4 d after the majority of cells have been generated in these regions of the spinal cord (Nornes and Das, 1974; Altman and Bayer, 1984) and less time after the majority have migrated from the central generative sites to their respective final locations. Development of these channels may occur on migrating cells, indicating that maturational aspects occur prior to a cell reaching its final location, as has been seen in neocortex (LoTurco and Kriegstein, 1991). It is clear that development of these channels occurs early in the cell's maturation process. The anatomic gradients of response development have also been seen in dissociated cells analyzed with a flow cytometric method (Schaffner et al., 1991). The ventrodorsal and rostrocaudal differences in appearance of the channels parallels the gradients of neurogenesis occurring in the spinal cord, where ventral cells are generated prior to dorsal cells and cervical prior to lumbosacral (Nornes and Das, 1974; Altman and Bayer, 1984). An aspect of interest, however, is that neurogenesis shows an approximately 3 d spread for the majority of cell generation, while the channel development gradient is shorter, on the order of 1–2 d. This may indicate that the dorsal cells develop channels more quickly after generation than do ventral cells.

Developmental changes are not seen during several hours in vitro *after dissociation*

The larger number of fields analyzed in the E15 lumbosacral experiments provided the opportunity to look for developmental changes occurring *in vitro* during the course of the experiment. There was no statistically significant progression in development of responses seen in either dorsal or ventral cells during the experiment. There may be several reasons for this. One is that the total time span elapsed was insufficient to see evidence of maturation. The approximately 5 hr separation between earliest and latest lumbosacral fields studied (roughly only 10% of the interage step size in the cervical experiments) may simply not be enough to allow for detecting additional maturation. Had greater lengths of time separation between fields or much greater numbers of fields been used, perhaps an *in vitro* maturation process could have been demonstrated. Another possibility is that when the cells are removed from their *in vivo* environment, they lose endogenous signals required to continue the maturation process. These signals for maturation could be of various sources: humoral substances from distant locations in the fetal environment, from other cells within the embryonic CNS, or perhaps influences mediated by direct cell-to-cell contact (Purves and Lichtman, 1985; Spitzer, 1991). Possibly the *in vitro* conditions were not permissive for the maturation to occur. After dissociation, the cells were placed in the culture dishes and allowed to adhere in a simple extracellular medium. There was no albumin or serum added, as well as no exogenous amino acids, vitamins, minerals, metabolites, or specific growth

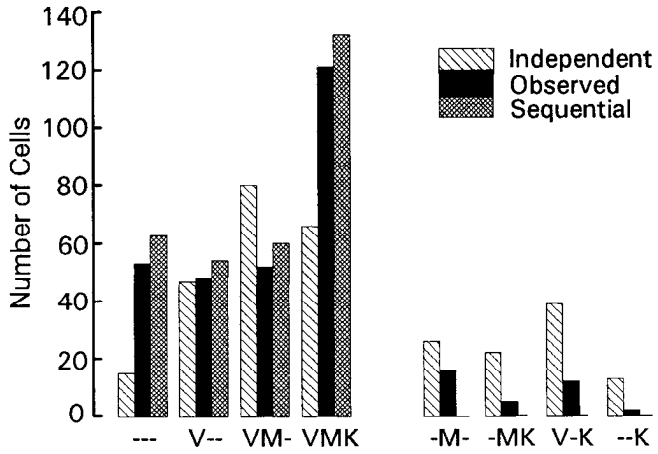

Figure 11. Creation of hypothetical distributions arising from the independent channel appearance hypothesis or the strictly sequential appearance hypothesis (see Results for method of generation of the values for each response category; category labeling as in Fig. 8). Response category distributions of E15 cervical dorsal cells are shown. The independent appearance hypothesized distribution is most divergent from the observed data for the categories of no responses (---), all three responses (*VMK*), and always places larger numbers of cells into the non-sequential-consistent (*rightmost*) group of categories. Sequential appearance hypothesized distribution is closer to the observed number for all categories (*except the single one of -M-*). It always has no cells in rightmost group of categories, which are "prohibited" under the strictly sequential hypothesis. The independent hypothesis is significantly different from the observed data by χ^2 statistic, and thus rejected, while the sequential development hypothetical distribution is not.

factors that are often included for extended periods of cell culture. It is possible that an intrinsic tendency of the cells to mature could have been inhibited by the absence of such components. However, the simplicity of the medium does not seem to have been acutely detrimental to the cells. Judging by the phase-contrast microscopic appearance of the cells, they remained healthy for over 12 and up to 24 hr after plating into culture dishes. Many cells would actively extend and rearrange processes, which would certainly require a degree of healthy function. Nevertheless, the absence of maturation *in vitro* substantiates the validity of grouping together all fields obtained from embryos of a particular age without regard to the time during the afternoon when the measurements were actually made.

This analysis also touches upon the topic of cell properties *in vivo* versus *in vitro*. Our evaluation of the cells occurred after a dissociation procedure that utilized papain. The question of possible alteration of cell properties due to the dissociation procedure is worth considering. In particular, could a cell's responsiveness have been lost or damaged during the dissociation procedure, and the responses measured in these experiments been as a result of some means of reconstitution of function? NMDA receptor responses have been shown to be lost due to several different enzymatic treatments (Akaike et al., 1988; Allen et al., 1988), while kainate responses were preserved after trypsin (Allen et al., 1988), as were sodium current, and GABA and kainate responses after collagenase and pronase exposure (Akaike et al., 1988). GABA responses were also preserved during exposure to papain (McCarren and Alger, 1987). Thus, it is not likely that the muscimol responses were altered by the dissociation procedure, and kainate responses seem more resistant to enzymatic loss than other forms of glutamate responsiveness. The analysis of the lumbosacral data indicated that no signifi-

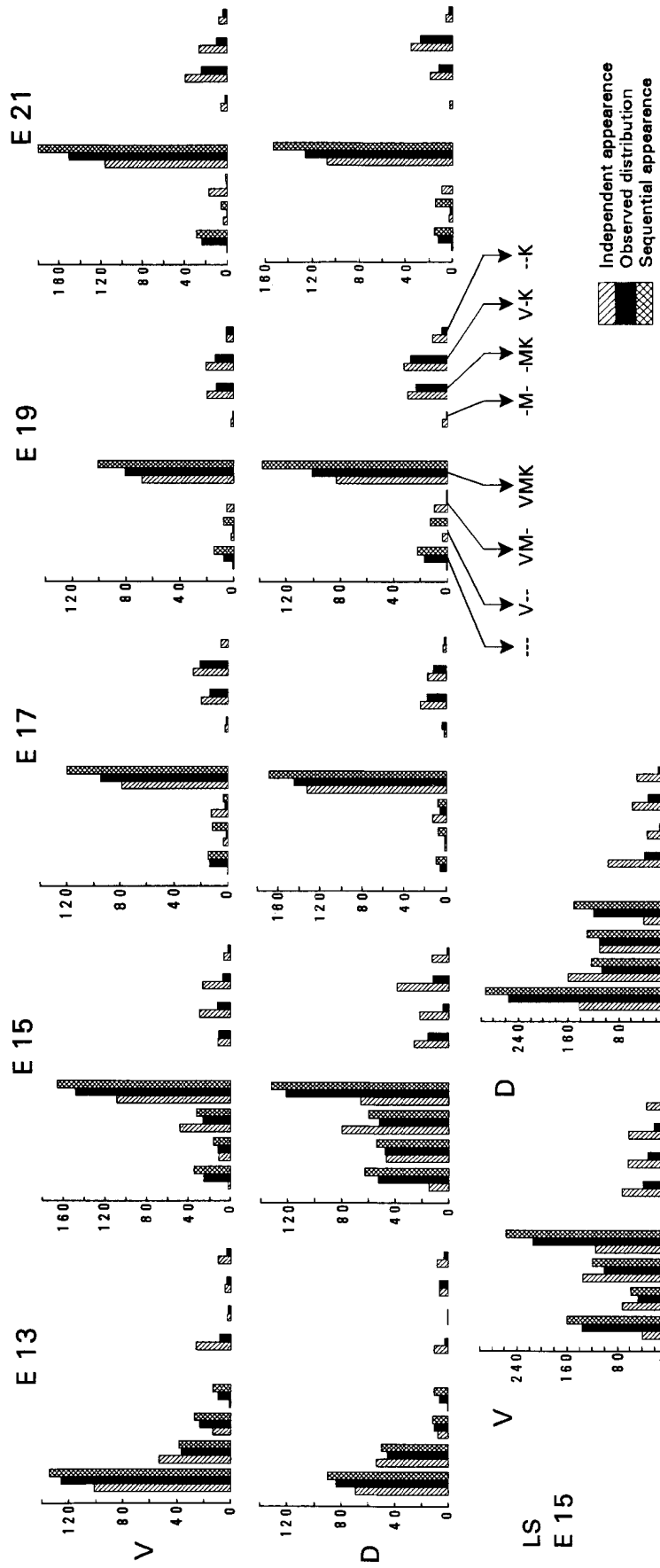

Figure 12. Most regions and ages allow rejection of the independent appearance of responses hypothesis but not the sequential appearance hypothesis. Hypothetical distributions of cells for all ages and regions examined were created and are shown as in Figure 11. The distributions generated by each hypothesis were tested against the observed distribution by calculating goodness-of-fit χ^2 statistics. The hypothesis of independent appearance of responses on each cell is rejected in all data sets except E17 cervical dorsal cells. The hypothesis of strictly sequential development with maintenance of responses is not rejectable in 8 of the 12 data sets. Only the E19 and E21 cervical dorsal and both lumbosacral (E15 ventral and dorsal) hypothesized distributions are different from the observed data.

cant increases in responses were seen during the recording period. If reconstitution of function were occurring, it would have had to occur largely within the first hour after dissociation, prior to beginning measurements on the cells. While we think it unlikely that the results seen actually reflect loss of responsiveness during dissociation and differences in ability to recover responses during the first hour after dissociation, we cannot absolutely rule this out.

With this caveat, the results demonstrated a sequence of channel expression in the early stages of cell maturation whereby a greater percentage of cells develop sodium channels than $GABA_A$-coupled channels, which in turn are present on a greater percentage than are kainate-activated channels. This relationship is true prior to the population distribution of responses reaching their plateau levels. This was clear for the regions as a whole, but the question of the sequence of channel development on each individual cell remained unclear.

Individual cells develop excitability in a stereotypic sequence

The issue considered was whether each cell matured in a manner stereotypic for all cells, or whether differences appeared in the cells with regard to the properties investigated here. This issue was approached in two ways. First, we assumed that there was not any stereotypic pattern of cell maturation. This implied that development of responsiveness on any particular cell occurred independently. At any particular age, a certain percentage of cells would have developed sodium channels, a certain percentage $GABA_A$-coupled channels, and a certain percentage kainate-activated channels, but whether or not a cell had developed one was independent of whether it had developed either or both of the others. This was tested and found to be statistically rejectable in all but one of the ages and regions examined. The one in which it was not rejectable (E17 cervical dorsal) was one with high response percentages for all three ligands, so that irrespective of any hypothesized distribution of responses, the majority of cells had to fall into a single response group, that is, that of responding to all three ligands. Thus, the E17 cervical dorsal cells are not a strong test of the hypothesis. It is the earlier embryonic cells that provide better tests because there are more cells in intermediate stages of differentiation, and these are the cells that are subject to greater variation in distribution depending on the hypothesis. In these data sets, as well as most of the older ages, independence of channel appearance is a rejectable hypothesis. Consequently, the proposition that the channels studied here are being expressed on cells based solely on independent timing can be rejected.

The second proposal was of the opposite extreme, and postulated that all cells exhibit an absolutely stereotyped pattern of maturation, that is, that of a strictly sequential development of channels. Sequential appearance of membrane properties has been shown for GABA and glutamate responses on cells in culture from several regions of rat CNS (Koller et al., 1990), and of sodium channels prior to NMDA receptors in rat cortex slices (LoTurco et al., 1991). In embryonic turtle cortex, however, the sequence is reversed, such that GABA responses appear prior to sodium conductance (Shen et al., 1988). In evaluating this hypothesis, it is again the younger ages that are more interesting because they contain the largest numbers of cells in intermediate stages. In this case, the hypothetical distribution could not be statistically rejected based on the observed data in 8 of the 12 ages and regions. This does not mean that the hypothesis is proven, only that it cannot be rejected on the basis of the data. There are, however, four sets of data in which the hypothesis was not consistent with the data. Two were in the two oldest cervical dorsal cells, and two were the dorsal and ventral lumbosacral data. These are best considered separately.

The two older sets where the sequential development hypothetical distribution was rejectable were the E19 and E21 cervical dorsal sets of data. At these ages, channel development plateaued in overall percentages, implying that the initial phases of maturation have concluded. It is to be expected, however, that other processes are still occurring in these cells. The hypothetical distribution was based not only on channels appearing in a strict sequence, but also on channels always remaining on the cells once they appear. If a type of channel developed in the appropriate sequence, but at a later stage became lost to analysis, the postulated distribution will not take this into account. Loss of response could occur for several reasons. One is that a channel developed during a cell's maturation may only transiently be present, and is eliminated when the cell enters a further differentiating phase of development (Bixby and Spitzer, 1984; Ito and Cherubini, 1991; Spitzer, 1991). Another reason may involve cells that are dying. Not all cells generated in the embryonic CNS survive into the adult period. Cell death is known to occur during the embryonic period *in vivo*, and includes cells from the pool of motoneurons (Lance-Jones, 1982; Oppenheim, 1986). If cells destined to die, or in the process of dying, lose channels or channel function, then they could add to the groups whose responses seem inconsistent with the sequential development postulate. Another possibility relates to the morphology of older cells and the dissociation procedure. Cells from older embryonic stages will have developed more extensive networks of axons and dendrites *in vivo*, and these are largely lost in the dissociation process. Only a minority of cells will have retained anything more than just a short initial segment of its processes after dissociation. If these cells had developed channels, but had a nonuniform distribution of channels in the membrane, placing most of its channels of one kind out on processes, then it is possible that after dissociation, insufficient numbers of channels would remain on the cell body to provide an adequate response. Either of these events could produce results as observed, and still have honored a principle of sequential development of channels on each cell.

An additional possible explanation for the disagreement between the observed and hypothetical distribution is that there may be a population of cells generated in the later embryonic period that do in fact mature in a manner that does not require the sequential appearance of each of the three channels examined here. Most of the neurons will have already been generated (Altman and Bayer, 1984) and moved through the early phases of cell maturation by the later ages examined. While the majority of spinal cord glia are generated postnatally (Gilmore, 1971; Ling, 1976), radial glia are present throughout the period studied, and during the later embryonic period, glial elements of the spinal cord are starting to accelerate their proliferation (Hirano and Goldman, 1988). Glia have in recent years been shown to express both voltage- and ligand-operated ion channels (Bormann and Kettenmann, 1988; Barres et al., 1990a,b), although the prevalence and purpose of these channels and receptors remain unclear. It is quite conceivable that their developmental process involves expression of different properties and at times different than the process followed by neurons. If so, then the glial cells may contribute to the response groups that are not

consistent with the sequential development of channels shown for the majority of cells.

The issues of cell death and generation of new cells during the embryonic period studied are relevant to another aspect of interpretation of this data. It is not possible to observe an individual cell continuously during its development through the ages investigated. Rather, we observed different cells individually at each of the ages studied. Our conclusions are inferences about what the development of each cell has been, and depend on several assumptions. One is that the cells evaluated at each age are largely still present at subsequent ages. Another assumption is that any cells added to the population evaluated at subsequent ages are similar in their time course of development with respect to these properties, so that the 2 d intervals used provide an equally fair evaluation of channel expression on all cells as they are added to the sampled cell population. Thus, our conclusions are inferences about an individual cell's development based on observation of different individual cells during embryonic development.

The other two sets of data where the hypothetical expected distribution was rejected, both E15 lumbosacral regions, are somewhat different because they are at a much earlier point in development. Many of the previous considerations are less likely to be significant in this case. These cells are in the early stages of cell maturation, and are less apt to have eliminated any channels that they had developed earlier following their final mitosis. There are not likely to be large amounts of cell death occurring in this region, or an extensive network of branching dendrites. This is also too early for large numbers of glia to be present in these regions. The difference in this case may relate to the larger numbers of these cells that were studied than in any category in the cervical regions (twice as many fields or more). This yields a more sensitive statistical test. Perhaps this is why the E15 lumbosacral data are not consistent with the hypothesis of strictly sequential appearance of channels while the E13 and E15 cervical data were indistinguishable between the hypothetical and observed.

Consequently, the conclusion to be drawn is that the hypothesis of all cells showing a strictly sequential development of the channels studied may not be absolutely valid, and that this is likely the case for both cervical and lumbosacral regions. The great majority of cells remain consistent with the principle of sequential development. However, the principle may not be an absolute requirement, but rather acts as a largely dominating influence on all cells. Alternatively, it may be that the majority of cells do completely follow the sequential development principle, but there is a small population of cells that do not adhere to the same developmental process. At present there is no way to distinguish between the two hypotheses, or to otherwise distinguish the small population of cells that do not follow the principle, if that is the case. Additional studies are necessary to elucidate further the processes occurring during early neuronal differentiation, and to discern the nature of any populations of cells that may show differences from the majority at an early point in the process. Extension of these studies to include other receptors and channels of importance to neuronal function and the differentiation process will also be valuable.

$GABA_A$ receptor activation is depolarizing in embryonic rat spinal cord cells

An additional aspect seen in this study and worth special note is that the responses seen to $GABA_A$ receptor activation were of a depolarizing nature. This is contrary to what would be expected in the majority of the mature CNS, where GABA is an inhibitory neurotransmitter and $GABA_A$ activation usually produces a stabilization of membrane potential at the resting potential or a mild hyperpolarization. The depolarizing $GABA_A$ response is not an entirely new phenomenon, and has been noted previously on dissociated spinal cord cells (Mandler et al., 1990). Depolarizing IPSPs have been seen in embryonic chick spinal cord (O'Donovan, 1989), as have depolarizing GABA responses in developing chick (Obata et al., 1978) and *Xenopus* (Bixby and Spitzer, 1984) spinal neurons. Depolarizing IPSPs or GABA responses are also present in immature hippocampus (Mueller et al., 1984; Cherubini et al., 1990). Consequently, while a depolarizing response is not a new finding, it is different from that found in mature spinal cord cells, where GABA functions primarily as an inhibitory transmitter. The mechanism of the depolarization in these cells has not been clearly determined. Our results showed that sodium entry was not necessary for this depolarization. Most other $GABA_A$ responses are largely chloride conductances, and a recent report indicates that this remains true for $GABA_A$ receptor responses in embryonic spinal cord cells (Kyrozis et al., 1992). Thus, it is possible that the response is due to a depolarizing chloride equilibrium potential in embryonic cells, but this has not been proven. It is not known when in development this response changes to an inhibitory one. In our rat spinal cord cells, the $GABA_A$ response remains depolarizing throughout the entire embryonic period. In chick spinal cord this response appears to have reversed in cells cultured from 10 d eggs (Obata et al., 1978). In hippocampus, it reverses around the second postnatal week (Mueller et al., 1984; Cherubini et al., 1991), but may persist as depolarizing in mature hippocampus on pyramidal cell dendrites (Avoli, 1992) and in interneurons (Michelson and Wong, 1991). It is not known what function a depolarizing response during the early maturation process serves.

The physiologic role of the channels studied here is particularly interesting. These channels and receptors appear very early in the cell's development, before widespread synaptogenesis has occurred (May and Biscoe, 1975; Saito, 1979; Blanton and Kriegstein, 1991; Cobas et al., 1991). In the adult CNS, these channels and receptors serve to conduct impulses to distant portions of a cell and to receive the synaptically transmitted impulse. These cellular properties may serve a different purpose in the embryonic period. Both GABA and glutamate receptor activation affect cell development in culture (Hansen et al., 1984; Moran and Patel, 1989; Patel et al., 1990). Cell membrane potential activity may also have effects on subsequent development (Shatz, 1990a,b; Agoston et al., 1991; Spitzer, 1991). There may be a broad principle that many substances that act as specific neurotransmitters in mature CNS appear in the developing CNS and help to direct development of the immature cells (Lauder, 1987; Mattson, 1988; Meier et al. 1991; Whitaker-Azmitia, 1991). If this is the case, perhaps the depolarizing responses to GABA in this period act as a stimulatory agent during development. This entire area remains open for further investigation.

References

Agoston DV, Eiden LE, Brenneman DE, Gozes I (1991) Spontaneous electrical activity regulates vasoactive intestinal peptide expression in dissociated spinal cord cell cultures. Mol Brain Res 10:235–240.

Akaike N, Kaneda M, Hori N, Krishtal OA (1988) Blockade of *N*-me-

thyl-D-aspartate response in enzyme-treated rat hippocampal neurons. Neurosci Lett 87:75–79.
Allen CN, Brady R, Swann J, Hori N, Carpenter DO (1988) *N*-methyl-D-aspartate (NMDA) receptors are inactivated by trypsin. Brain Res 458:147–150.
Altman J, Bayer SA (1984) The development of the rat spinal cord. New York: Springer.
Apell H-J, Bersch B (1987) Oxonol VI as an optical indicator for membrane potentials in lipid vesicles. Biochim Biophys Acta 903: 480–494.
Avoli M (1992) Synaptic activation of $GABA_A$ receptors causes a depolarizing potential under physiological conditions in rat hippocampal pyramidal cells. Eur J Neurosci 4:16–26.
Baccaglini PI, Spitzer NC (1977) Developmental changes in the inward current of the action potential of Rohon-Beard neurones. J Physiol (Lond) 271:93–117.
Barres BA, Chun LLY, Corey DP (1990a) Ion channels in vertebrate glia. Annu Rev Neurosci 13:441–471.
Barres BA, Koroshetz WJ, Swartz KJ, Chun LLY, Corey DP (1990b) Ion channel expression by white matter glia: the O-2A glial progenitor cell. Neuron 4:507–524.
Bashford CL, Alder GM, Gray MA, Micklem KJ, Taylor CC, Turek PJ, Pasternak CA (1985) Oxonol dyes as monitors of membrane potential: the effect of viruses and toxins on the plasma membrane potential of animal cells in monolayer culture and in suspension. J Cell Physiol 123:326–336.
Beckh S, Pongs O (1990) Members of the RCK potassium channel family are differentially expressed in the rat nervous system. EMBO J 9:777–782.
Beckh S, Noda M, Lubbert H, Numa S (1989) Differential regulation of three sodium channel messenger RNAs in the rat central nervous system during development. EMBO J 8:3611–3616.
Bixby JL, Spitzer NC (1982) The appearance and development of chemosensitivity in Rohon-Beard neurones of the *Xenopus* spinal cord. J Physiol (Lond) 330:513–536.
Bixby JL, Spitzer NC (1984) The appearance and development of neurotransmitter sensitivity in *Xenopus* embryonic spinal neurons *in vitro*. J Physiol (Lond) 353:143–155.
Blanton MG, Kriegstein AR (1991) Appearance of putative amino acid neurotransmitters during differentiation of neurons in embryonic turtle cerebral cortex. J Comp Neurol 310:571–592.
Blanton MG, LoTurco JJ, Kriegstein AR (1990) Endogenous neurotransmitter activates *N*-methyl-D-aspartate receptors on differentiating neurons in embryonic cortex. Proc Natl Acad Sci USA 87:8027–8030.
Bormann J, Kettenmann H (1988) Patch-clamp study of GABA receptor Cl^- channels in cultured astrocytes. Proc Natl Acad Sci USA 85:9336–9340.
Brauner T, Hulser DF, Strasser RJ (1984) Comparative measurements of membrane potentials with microelectrodes and voltage-sensitive dyes. Biochim Biophys Acta 771:208–216.
Bregman BS (1987) Development of serotonin immunoreactivity in the rat spinal cord and its plasticity after neonatal spinal cord lesions. Dev Brain Res 34:245–263.
Brenneman DE, Forsythe ID, Nicol T, Nelson PG (1990) *N*-methyl-D-aspartate receptors influence neuronal survival in developing spinal cord cultures. Dev Brain Res 51:63–68.
Cherubini E, Rovira C, Gaiarsa JL, Corradetti R, Ben Ari Y (1990) GABA mediated excitation in immature rat CA3 hippocampal neurons. Int J Dev Neurosci 8:481–490.
Cherubini E, Gaiarsa JL, Ben-Ari Y (1991) GABA: an excitatory transmitter in early postnatal life. Trends Neurosci 14:515–519.
Civitelli R, Reid IR, Halstead LR, Avioli LV, Hruska KA (1987) Membrane potential and cation content of osteoblast-like cells (UMR 106) assessed by fluorescent dyes. J Cell Physiol 131:434–441.
Clarke RJ (1991) Binding and diffusion kinetics of the interaction of a hydrophobic potential-sensitive dye with lipid vesicles. Biophys Chem 39:91–106.
Cobas A, Fairen A, Alvarez-Bolado G, Sanchez MP (1991) Prenatal development of the intrinsic neurons of the rat neocortex: a comparative study of the distribution of GABA-immunoreactive cells and the GABA-A receptor. Neuroscience 40:375–397.
Dasheiff RM (1988) Fluorescent voltage-sensitive dyes: applications for neurophysiology. J Clin Neurophysiol 5:211–235.
Fuji K, Senba E, Fujii S, Nomura I, Wu J-Y, Ueda Y, Tohyama M (1985) Distribution, ontogeny and projections of cholecystokinin-8, vasoactive intestinal polypeptide and γ-aminobutyrate-containing neurons systems in the rat spinal cord: an immunohistochemical analysis. Neuroscience 14:881–894.
Gilmore SA (1971) Neuroglial population in the spinal white matter of neonatal and early postnatal rats: an autoradiographic study of numbers of neuroglia and changes in their proliferative activity. Anat Rec 171:283–292.
Hansen GH, Meier E, Schousboe A (1984) GABA influences the ultrastructure composition of cerebellar granule cells during development in culture. Int J Dev Neurosci 2:247–257.
Hebel R, Stromberg MW (1986) Anatomy and embryology of the laboratory rat. Worthsee, Germany: BioMed.
Hirano M, Goldman JE (1988) Gliogenesis in rat spinal cord: evidence for origin of astrocytes and oligodendrocytes from radial precursors. J Neurosci Res 21:155–167.
Huettner JE, Baughman RW (1986) Primary culture of identified neurons from the visual cortex of postnatal rats. J Neurosci 6:3044–4060.
Ito S, Cherubini E (1991) Strychnine-sensitive glycine responses of neonatal rat hippocampal neurones. J Physiol (Lond) 440:67–83.
Koller H, Siebler M, Schmalenbach C, Muller H-W (1990) GABA and glutamate receptor development of cultured neurons from rat hippocampus, septal region and neocortex. Synapse 5:59–64.
Kyrozis A, Reichling DB, MacDermott AB (1992) Depolarization of cultured embryonic rat spinal cord neurons by $GABA_A$ receptor activation: magnitude and ionic basis. Soc Neurosci Abstr 18:980.
Lance-Jones C (1982) Motoneuron cell death in the developing lumbar spinal cord of the mouse. Dev Brain Res 4:473–479.
Lauder JM (1987) Neurotransmitters as morphogenetic signals and trophic factors. In: Model systems of development and aging of the nervous system (Vernadakis A, ed), pp 219–237. Boston: Nijhoff.
Ling EA (1976) Study in the changes of the proportions and numbers of the various glial cell types in the spinal cord of neonatal and young adult rats. Acta Anat 96:188–195.
LoTurco JJ, Kriegstein AR (1991) Clusters of coupled neuroblasts in embryonic neocortex. Science 252:563–566.
LoTurco JJ, Blanton MG, Kriegstein AR (1991) Initial expression and endogenous activation of NMDA channels in early neocortical development. J Neurosci 11:792–799.
Mandler RN, Schaffner AE, Novotny EA, Lange GD, Smith SV, Barker JL (1990) Electrical and chemical excitability appear one week before birth in the embryonic rat spinal cord. Brain Res 522:46–54.
Marti E, Gibson SJ, Polak JM, Facer P, Springall DR, Van Aswegen G, Aitchison M, Koltzenburg M (1987) Ontogeny of peptide- and amine-containing neurones in motor, sensory, and autonomic regions of rat and human spinal cord, dorsal root ganglia, and rat skin. J Comp Neurol 266:332–359.
Mattson MP (1988) Neurotransmitters in the regulation of neuronal cytoarchitecture. Brain Res Rev 13:179–212.
May MK, Biscoe TJ (1975) An investigation of the foetal rat spinal cord. II. An ultrastructural study on the development of synapses with the aid of observations on some electrophysiological properties. Cell Tissue Res 158:251–268.
McCarren M, Alger BE (1987) Papain effects on rat hippocampal neurons in the slice preparation. Neurosci Lett 78:307–310.
McCobb DP, Best PM, Beam KG (1989) Development alters the expression of calcium currents in chick limb motoneurons. Neuron 2:1633–1643.
McCobb DP, Best PM, Beam KG (1990) The differentiation of excitability in embryonic chick limb motoneurons. J Neurosci 10:2974–2984.
Meier E, Hertz L, Schousboe A (1991) Neurotransmitters as developmental signals. Neurochem Int 19:1–15.
Michelson HB, Wong RKS (1991) Excitatory synaptic responses mediated by $GABA_A$ receptors in the hippocampus. Science 253:1420–1423.
Moran J, Patel AJ (1989) Stimulation of *N*-methyl-D-aspartate receptor promotes the biochemical differentiation of cerebellar granule neurons and not astrocytes. Brain Res 486:15–25.
Mueller AL, Taube JS, Schwartzkroin PA (1984) Development of hyperpolarizing inhibitory postsynaptic potentials and hyperpolarizing response to γ-aminobutyric acid in rabbit hippocampus studied *in vitro*. J Neurosci 4:860–867.
Nornes HO, Das GD (1974) Temporal pattern of neurogenesis in spinal cord of rat. I. An autoradiographic study—time and sites of

origin and migration and settling patterns of neuroblasts. Brain Res 73:121–138.

Obata K, Oide M, Tanaka H (1978) Excitatory and inhibitory actions of GABA and glycine on embryonic chick spinal neurons in culture. Brain Res 144:179–184.

O'Donovan MJ (1989) Motor activity in the isolated spinal cord of the chick embryo: synaptic drive and firing pattern of single motoneurons. J Neurosci 9:943–958.

Oppenheim RW (1986) The absence of significant postnatal motoneuron death in the brachial and lumbar spinal cord of the rat. J Comp Neurol 246:281–286.

Patel AJ, Hunt A, Sanfeliu C (1990) Cell-type specific effects of *N*-methyl-D-aspartate on biochemical differentiation of subcortical neurons in culture. Int J Dev Neurosci 8:379–389.

Plasek J, Hrouda V (1991) Assessment of membrane potential changes using the carbocyanine dye, diS-C_3-(5): synchronous excitation spectroscopy studies. Eur Biophys J 19:183–188.

Poulter MO, Barker JL, O'Carroll A-M, Lolait SJ, Mahan LA (1992) Differential and transient expression of $GABA_A$ receptor α-subunit mRNAs in the developing rat CNS. J Neurosci 12:2888–2900.

Purves D, Lichtman JW (1985) Principles of neurodevelopment. Sunderland, MA: Sinauer.

Saito K (1979) Development of spinal reflexes in the rat fetus studied *in vitro*. J Physiol (Lond) 294:581–594.

Schaffner AE, Smith SV, Barker JL (1991) Electrical and chemical excitability develop along anatomical gradients in the embryonic rat spinal cord. Soc Neurosci Abstr 17:749.

Shatz CJ (1990a) Competitive interaction between retinal ganglion cells during prenatal development. J Neurobiol 21:197–211.

Shatz CJ (1990b) Impulse activity and the patterning of connections during CNS development. Neuron 5:745–756.

Shen JM, Huguenard JR, Kriegstein AR (1988) Development of GABA responsiveness in embryonic turtle cortical neurons. Neurosci Lett 89:335–341.

Spitzer NC (1991) A developmental handshake: neuronal control of ionic currents and their control of neuronal differentiation. J Neurobiol 22:659–673.

Waggoner AS (1979) Dye indicators of membrane potential. Annu Rev Biophys Bioeng 8:47–68.

Walton MK, Schaffner AE, Barker JL (1990) Development of membrane responses of embryonic rat spinal cord cells. Soc Neurosci Abstr 16:272.17.

Whitaker-Azmitia PM (1991) Role of serotonin and other neurotransmitter receptors in brain development: basis for developmental pharmacology. Pharmacol Rev 43:553–561.

Wilson HA, Chused TM (1985) Lymphocyte membrane potential and Ca^{2+}-sensitive potassium channels described by oxonol dye fluorescence measurements. J Cell Physiol 125:72–81.

Wolff JR, Bottcher H, Zetzsche T, Oertel WH, Chronwall BM (1984) Development of GABAergic neurons in rat visual cortex as identified by glutamate decarboxylase-like immunoreactivity. Neurosci Lett 47: 207–212.