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Starburst amacrine cells change from spiking to nonspiking neurons during retinal development

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ABSTRACT **The membrane excitability of cholinergic (starburst) amacrine cells was studied in the rabbit retina during postnatal development. Whole-cell patch-clamp recordings were made from 110 displaced starburst cells in a thin retinal slice preparation of rabbits between postnatal days P1 and P56 old. We report that displaced starburst cells undergo a dramatic transition from spiking to nonspiking, caused by a loss of voltage-gated Na currents. This change in membrane excitability occurred just after eye opening (P10), such that all of the starburst cells tested before eye opening had conspicuous tetrodotoxin-sensitive Na currents and action potentials, but none tested after the first 3 postnatal weeks had detectable Na currents or spikes. Our results suggest that starburst cells use action potentials transiently during development and probably play a functional role in visual development. These cells then cease to spike as the retina matures, presumably consistent with their role in visual processing in the mature retina.**

In the vertebrate retina, synaptic transmission by most interneurons is mediated by nonspiking, graded membrane potentials (1). Action potentials so far have been found in only a relatively small population of retinal interneurons, predominantly wide-field amacrine cell types (e.g., refs. 2–10). Many of the nonspiking retinal interneurons are believed to be electrotonically compact and thus do not require action potential propagation for synaptic integration or neurotransmitter release. However, there are other retinal interneurons, e.g., some axon-bearing horizontal cells, that respond to illumination of the retina with sustained graded potentials, but their electrotonic structure does not appear to be compact. It has been suggested that action potential propagation in these rather extensive interneurons may be inappropriate for specific functional reasons. For example, it has been thought that attenuation of synaptic signals in some of these nonspiking cells may serve to keep different parts of the cell functionally segregated (11).

The cholinergic (starburst) amacrine cell is a medium-field amacrine cell type found in all mammalian retinas examined so far (for review, see refs. 12–14). Starburst cells exist in the rabbit retina as two mirror-symmetric populations in the inner plexiform layer (IPL), each having radially symmetric dendritic fields that extend from 200 to 800 μm in diameter depending on eccentricity (15–17). These cells receive input from cone bipolar cells and other amacrine cells and make direct output synapses onto ganglion cells and amacrine cells, including starburst cells themselves (18–21). It has been proposed that starburst cells may be involved in the production of directional selectivity and in the regulation of the responsiveness of ganglion cells in the retina (e.g., refs. 13, 22, and 23). It has been further postulated that the starburst dendritic tree consists of semiisolated local processing units, which may serve as a substrate for synaptic interactions crucial for directional selectivity (23). In order for these local units to function autonomously, it has been thought that spike propagation in the starburst cell would be inappropriate.

Experimental results on starburst cell excitability have so far remained inconsistent. Whereas previous studies using intracellular (9) and extracellular (24) recordings have suggested spikes from starburst cells in the adult rabbit, recent patch-clamp recordings have not been able to find any evidence for regenerative activities in these cells (25, 26). Given the importance of this subject to our understanding of the functional role of the starburst amacrine and other medium-field amacrine cell types, it is important to determine if the starburst amacrine cell is indeed capable of generating somatic action potentials.

In addition to a possible role for action potentials in signal processing, it seems possible that membrane excitability could also play a role in the developing retina (27–29). Amacrine cells and ganglion cells are the first cell types to differentiate during development in rabbit (30, 31), and displaced starburst cells in the rabbit retina already attain their adult-like dendritic morphology at birth (32). It is known that ganglion cells in the developing retina fire correlated spontaneous action potentials that are believed to play an important role in establishing precise neuronal connectivity in the visual pathway (33–35). There is some evidence that amacrine cells (35) and in particular starburst amacrines (52) may participate in generating spontaneous activity.

In this study, we examined the membrane excitability of starburst amacrine cells in the rabbit retina during postnatal development. We report that displaced starburst cells undergo a transition from spiking to nonspiking during a crucial period in visual development. This change in membrane excitability is caused by a loss of Na channel current just after eye opening. Thus, starburst cells may use action potentials transiently during a specific period in development, as a part of a network that generates the spontaneous activity. They then become nonspiking neurons as the retina matures, presumably consistent with their role in visual processing in the mature retina.

MATERIALS AND METHODS

Whole-Cell Patch-Clamp Recording in Rabbit Retinal Slices. Retinal slices were prepared from pigmented rabbits aged between P1 and P56 (P0, day of birth) as described (36). Briefly, 3- to 5-mm wide strips of retina, typically from areas within 6 mm from the visual streak in the inferior retina, were isolated on nitrocellulose filter paper (Millipore type HAWP) and sliced at a thickness of 150–200 μm in a cold (4°C) physiological saline containing 137 mM NaCl, 4.3 mM KCl, 1.7 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM Hepes, 5 mM glucose (pH 7.4 with NaOH). The slices were subsequently incubated in Ames medium (Sigma; ref. 37) that was equilibrated with carbogen (95% O_2/5% CO_2) for 1–10 hr at room temperature (21–25°C).

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Abbreviations: GCL, ganglion cell layer; IPL, inner plexiform layer; DAPI, 4′,6-diamidino-2-phenylindole; TTX, tetrodotoxin.

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Whole-cell patch-clamp recordings were made from slices under a fixed-stage upright microscope (Zeiss) equipped with epifluorescence and a 40× water-immersion objective lens configured for Hoffman modulation optics (Modulation Optics, Greenvale, NY). Cells were recorded in carbogenated Ames medium at room temperature (21–25°C) with 2–5-MΩ resistance electrodes containing 95 mM KCl, 10 mM NaCl, 0.5 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM EGTA, 10 mM Hepes, 2 mM adenosine 5′-triphosphate (ATP-sodium salt, Sigma), 0.5 mM guanosine 5′-triphosphate (GTP-sodium slat, Sigma), and 1 mM ascorbate (pH 7.2 with KOH). Series resistance, typically between 10 and 15 MΩ, was compensated by 40–70% with the series resistance compensation circuitry in the patch-clamp amplifier (Axopatch 200A, Axon Instruments, Burlingame, CA). The liquid junction potential was corrected as described (36, 38). Data acquisition and analysis were done with the software programs PCLAMP (Axon Instruments) and ORIGIN (MicroCal, Northampton, MA).

Identification of Displaced Starburst Cells in the Slice. Identification of a displaced starburst cell was based on its round, 7–10-μm cell body near the border between ganglion cell layer (GCL) and inner nuclear layer and on its thin, unistratified dendritic morphology, which was always examined after whole-cell patch-clamp with a Lucifer yellow-filled pipette. In rabbits older than P10, the identification of displaced starburst cells was further assisted by the selective staining pattern of the fluorescent dye 4′,6-diamidino-2-phenylindole (DAPI, 0.1–0.5 μg) injected intraocularly 1–2 days before the experiments (36, 39). DAPI injection was not attempted in rabbits younger than P10, due to its lack of selectivity for displaced starburst cells; however, displaced starburst cells recorded from these younger rabbits had cell bodies of similar diameter and a dendritic morphology similar to those stained with DAPI (see *Results*), though fine dendritic spines were observed predominantly from cells in young animals during the first postnatal week as previously reported (32). In general, we found the dendritic diameter of starburst cells increased with animal age during the first 2–3 postnatal weeks, although the dendrites as revealed by the staining of Lucifer yellow varied considerably in length from cell to cell, presumably due to differences in eccentricity and in the degree of dendritic damage after slicing. In young retinas without the DAPI label, we occasionally also encountered some axonless cells in the GCL, with a somal size similar to that of the starburst cells, and with dendrites stratifying rather narrowly in the sublaminar b of the IPL, perhaps corresponding to the nonstarburst amacrine cell previously described (32). However, this cell type had a much larger dendritic field and somewhat thicker proximal dendrites than those of starburst cells and was, therefore, quite easily distinguished from displaced starburst cells. Although we cannot exclude the possibility that in young rabbits without DAPI injection some other axonless nonstarburst cells but with a similar dendritic morphology in the slice might have been included within our sample, we believe that our results would remain the same regardless of whether there were an occasional misidentification of cell type, because every displaced starburst cell we recorded from in the young retina had prominent Na currents and characteristic single-spike action potential activity, whereas none of the DAPI-labeled starburst cells from animals older than P28 had action potentials (see *Results*).

RESULTS

Displaced Starburst Cells in Postnatal Retinal Slices. We recorded from 110 displaced starburst cells from rabbits aged

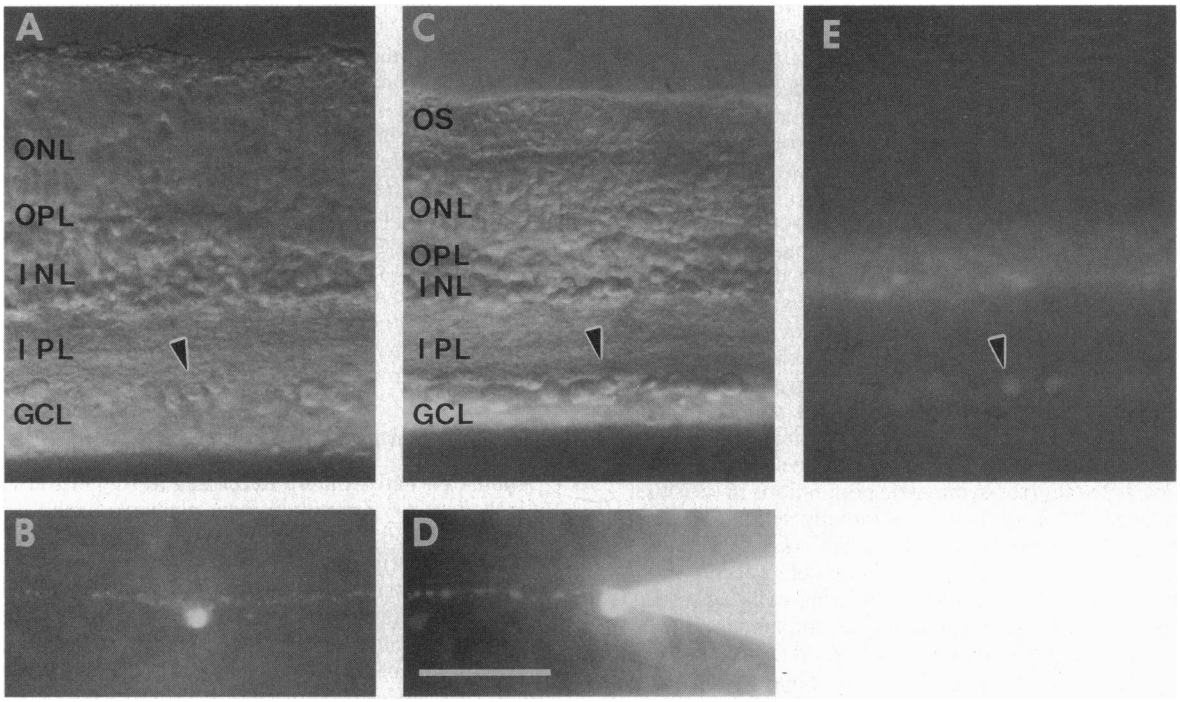

FIG. 1. Photomicrographs of rabbit retinal slices. (*A*) At P8, GCL and the inner nuclear layer (INL) have a morphology like that in the mature retina, but the outer nuclear layer (ONL) is still in the process of cellular differentiation. Photoreceptor inner segments and outer segments are just detectable near the upper edge of the slice. The arrowhead points to a displaced starburst cell near the border between the IPL and GCL. OPL, outer plexiform layer. (See ref. 40 for a more detailed morphological description of the postnatal rabbit retina.) (*B*) Epifluorescence photograph of cell indicated by the arrowhead in *A*, showing starburst dendritic morphology after whole-cell patch-clamp with a Lucifer yellow-filled pipette. (*C*) A P18 retinal slice with features nearly indistinguishable from those of adult rabbits. Displaced starburst cells in this retina were selectively labeled by the fluorescent dye DAPI. The arrowhead points to same DAPI-stained displaced starburst cell as shown in *D* and *E*. (*D*) Fluorescent micrograph (with a filter set for Lucifer yellow) of starburst cell indicated by the arrowhead in *C*, after filling with Lucifer yellow. (*E*) Fluorescence micrograph (with a filter set for DAPI) of the same slice as in *C* and *D*, showing selective staining of displaced starburst cells in the GCL. (Bar = 50 μm.)

between postnatal days P1 and P56 in a retinal slice preparation (36). As previously reported (31, 40), we found that maturation of the rabbit retina occurred mainly during the first 3 postnatal weeks. At birth, cells in the ganglion cell layer of the retinal slice appeared well differentiated (data not shown). According to Wong and Collin (32), displaced starburst amacrine cells already attain their distinctive morphology, and the basic dendritic branching pattern of displaced starburst cells remains similar throughout postnatal development, though the size of the starburst dendritic field grows progressively as the retina matures. Fig. 1 shows displaced starburst cells and retinal slices prepared from P8 and P18 retinas. At P8, the retina was still immature: cells in the outer nuclear layer were still in the process of differentiation, and photoreceptor outer segments were barely detectable, though the GCL and the inner nuclear layer already appeared fully differentiated. At P18, cellular differentiation appeared to have been completed in all retinal layers. The retina had also reduced its thickness significantly and appeared nearly indistinguishable from adult retina (Fig. 1*C*).

As shown in Fig. 1 *B* and *D*, displaced starburst amacrine cells in P8 and P18 retinal slices shared a similar, distinctive morphology. They had a nearly round, 7–10-μm cell body in the GCL, near the border between the GCL and the IPL. These cells lack axons and have a similar branching structure, with dendrites ramifying narrowly in sublaminar b of the IPL. These dendrites were thin and, in the more mature rabbits, contained many varicosities in distal regions. Fine dendritic spines were observed mainly in young rabbits during the first postnatal week (data not shown). Fig. 1*E* shows the epifluorescence micrograph of the DAPI staining pattern in the same P18 retinal slice as shown in Fig. 1 *C* and *D*. Displaced starburst amacrine cells were selectively stained by DAPI and were identified before patch-clamp recording.

Responses of Starburst Cells Before Eye Opening. Displaced starburst cells were recorded in Ames medium under both current-clamp and voltage-clamp conditions with pipettes filled mainly with KCl as the charge carrier (see *Materials and Methods* for composition). The resting membrane potential of these cells, measured as the zero current potential immediately after the formation of the whole-cell recording, was usually between −75 mV and −45 mV. Cells were first held under current clamp at around −80 mV with a constant hyperpolarizing current. Membrane voltage responses were then evoked with depolarizing current pulses of 50–70-ms duration and 100–250-pA amplitude.

Displaced starburst cells recorded from animals before eye opening (<P10) were consistently electrically excitable, responding to sustained depolarizing current pulses with prominent action potentials (Fig. 2*A*, P2 and P8). This regenerative activity had a voltage threshold between −45 and −35 mV, which appeared to be higher than that for most of the ganglion cells recorded under similar conditions. Action potentials in these starburst cells usually consisted of a single spike, although on rare occasions we also observed additional repetitive spikes that were smaller and of lower threshold, perhaps originating from dendrites (see below). Regenerative activities could also be elicited from these cells with short (0.5–5-ms duration), depolarizing current pulses, and action potentials evoked under such conditions always contained a single spike (Fig. 3*A*). Although the action potential usually had a larger amplitude when the cell was held at more hyperpolarized potentials (e.g., −80 mV), action potentials could also be elicited when the cell was held near to its resting membrane potential (e.g., −60 mV). Spontaneous firing was usually not observed (except in one cell), but this could have been the result of the preparation of the slice for recording.

When recorded under voltage clamp (Fig. 2B, P2 and P8), these cells showed prominent transient inward currents, which were activated usually at test potentials above −50 mV and reached a maximum peak amplitude at around −15 mV. In most cases, a net-inward current could be detected before

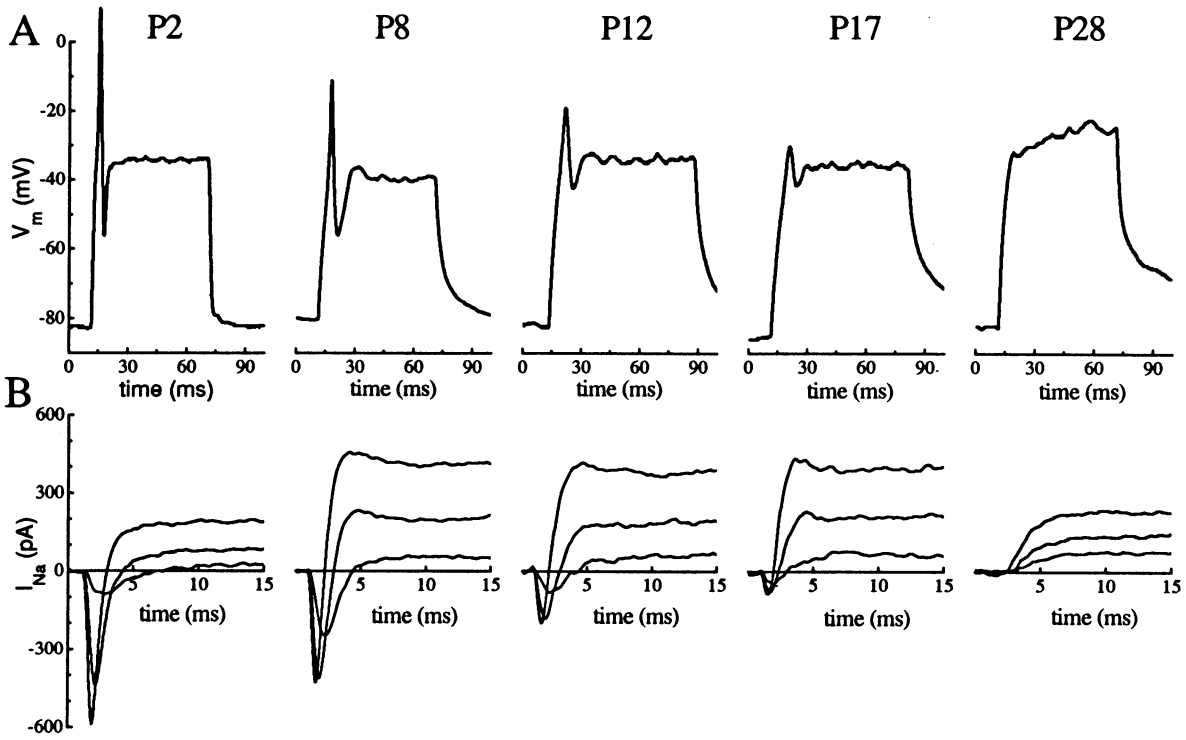

FIG. 2. Action potentials and Na currents from displaced starburst cells. (*A*) Membrane potential responses to depolarizing current pulses (50–70-ms long, 100–250 pA in amplitude) under current-clamp in cells from rabbits aged P2, P8, P12, P17, and P28. Cells were held near −80 mV by a constant holding current. The resting membrane potential ranged from −75 to −45 mV. (*B*) Voltage-clamp records from same cells. Currents were elicited by voltage steps from a holding potential (V_h) of −70 mV to test potentials of −30, −20, and −10 mV, and were corrected for membrane leak and capacitive transients.

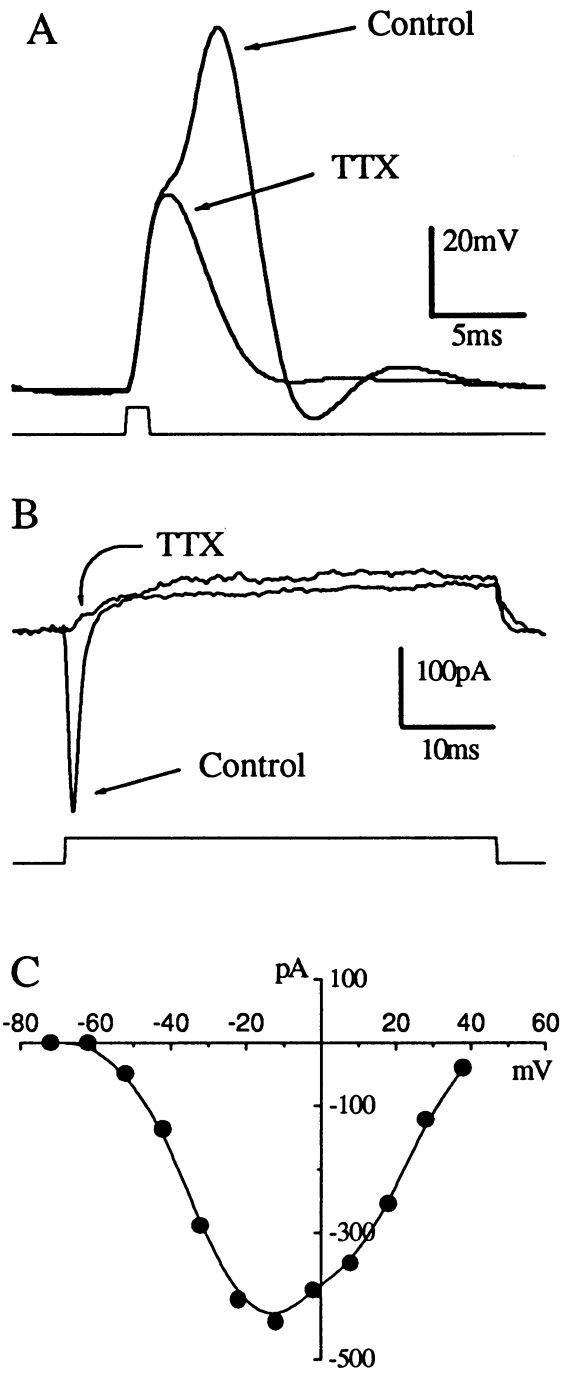

FIG. 3. (*A*) Action potential from a P2 starburst cell, elicited by a 0.6-ms-long current pulse (bottom trace). TTX (300 nM) completely blocked the action potential. (*B*) Na current elicited from the same cell by a voltage step from −70 to −20 mV (bottom trace), blocked by 300 nM TTX. Nearly all of the transient inward currents in starburst cells were sensitive to TTX, and voltage-gated Ca currents were negligibly small under our recording conditions. The TTX effect on both action potential and Na current was completely reversible after wash-out (data not shown). (*C*) Current–voltage (*I–V*) curve for peak Na currents measured after leak subtraction from a starburst cell in a P8 retinal slice (V_h = −82 mV).

corrections for membrane leakage or capacitive transients were made. In general, the transient inward current appeared reasonably well voltage-clamped, with activation and inactivation completed within 5 ms of the onset of depolarizing voltage pulses. Fig. 3*C* shows the current-voltage relation of the transient, inward currents from a P8 starburst cell voltage-clamped from a holding potential of −82 mV. The peak current amplitude was measured after leak subtraction. Both the regenerative spikes evoked under current clamp and the transient inward currents recorded under voltage clamp could be blocked reversibly by 300 nM tetrodotoxin (TTX) (Fig. 3 *A* and *B*). They were also abolished when Na^+ in the extracellular saline was replaced by choline (data not shown), indicating that voltage-gated, TTX-sensitive Na currents in starburst cells were responsible for the production of these spikes and transient inward currents.

Occasionally, we also recorded poorly-clamped transient inward currents, with delayed activation and repetitive current peaks in response to a single voltage step. These currents presumably originated from remote regions of the dendrites. Fig. 4 shows a current-clamp recordings from a starburst cell in a P16 retinal slice. Small repetitive minispikes were observed when the cell was depolarized to above −55 mV. These repetitive spikes, as well as the small repetitive inward current peaks seen from the same cell under voltage-clamp (data not shown), were reversibly blocked by 500 nM TTX. Repetitive minispikes were rarely seen (in two cells), but poorly voltage-clamped Na currents, often with small repetitive peaks, have been seen more frequently, particularly from starburst cells of 2- to 3-week-old rabbits.

Transition in Membrane Excitability After Eye Opening. At eye opening (P10-P11), a time when visual events would begin to emerge and the correlation in spontaneous activity to subside in the retina (5, 41–44), we found that starburst cells showed a striking transition in their membrane excitability. The amplitude of their action potentials began to decrease and some cells lost their spiking capability. This decline in membrane excitability occurred rather rapidly, such that by P17, only about 30% of the starburst cells tested showed a small regenerative response to depolarizing current injection (Fig. 2, P17). Many of these small regenerative responses could be resolved only after a close inspection of the voltage charging curve, which showed a TTX-sensitive inflection point in its rising phase. By the fourth postnatal week, spikes could no longer be observed (Fig. 2*A*, P28). These changes in electrical activity were mirrored by changes in Na current amplitude, which decreased progressively with age after eye opening and eventually disappeared entirely. Fig. 2*B* shows voltage-clamp recordings from the same cells shown in Fig. 2*A*. Whole-cell currents were elicited from a holding potential of −70 mV to test potentials of −30, −20, and −10 mV, and were corrected for membrane leakage and capacitive transients. Na currents were most prominent in young animals before eye opening, and began to decrease in amplitude dramatically after P8, though there was no obvious change in current activation or inactivation kinetics (data not shown). We also did not notice any dramatic, age-dependent changes in potassium currents in the starburst cells, though potassium currents with both fast and slowly inactivating components did vary in amplitude from cell to cell.

Fig. 5 summarizes the results we obtained from all of the starburst cells recorded under voltage-clamp at a holding potential (V_h) of −70 mV (similar results were also obtained at a V_h below −80 mV). We show as a function of animal age the percentage of cells with transient inward currents (Fig. 5*A*), and the mean peak-amplitude of the current from cells for which Na currents were observed (Fig. 5*B*). The peak amplitude of transient inward currents was measured after leak subtraction as an estimate of the size of Na currents in these cells. Because the cells were recorded in solutions that resembled physiological ionic concentrations, the inward currents were possibly contaminated by currents other than voltage-gated Na currents. However, the transient inward currents were nearly completely blocked by TTX (see Fig. 3 *A* and *B*), and voltage-gated Ca currents in these cells were small in physiological solutions (data not shown). A cell was determined to have Na currents if the maximum transient inward

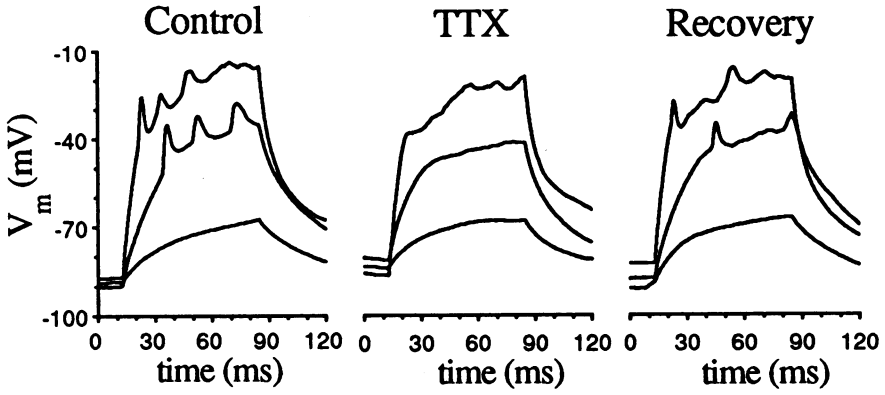

FIG. 4. Membrane voltage responses of a P16 starburst cell to depolarizing current pulses of 10, 30, and 70 pA, respectively. Small, repetitive spikes are clearly seen on the voltage charging curves under current-clamp. These minispikes were reversibly blocked by 500 nM TTX.

current was larger than 15 pA. These data show a clear transition at about P11-P14: shortly after eye-opening, displaced starburst cells lost their ability to generate action potentials, and their Na currents declined from a level of about 250–350 pA before eye opening to a level of <100 pA immediately after. The residual 50–100 pA of Na current, which already had too little contribution to the net inward current to generate a significant regenerative membrane response, remained in about 50% of the cells during the third postnatal week and eventually disappeared completely by the fourth postnatal week. Thus, displaced starburst amacrine cells lost their spiking capability after eye opening. We presume this reflects a decrease in the expression of Na channels in displaced starburst cells, though other possibilities (e.g., channel inactivation) cannot at present be excluded.

DISCUSSION

Our results suggest that displaced starburst cells in the mature rabbit retina do not fire TTX-sensitive somatic action potentials. This is in agreement with previous reports of patch-clamp studies of the starburst cell in the whole-mount rabbit retina (25, 26). Although our data still cannot explain the discrepancy between results obtained by patch-clamp recordings and by intracellular (9) and extracellular (24) recordings, our results argue against the possibility that the lack of somatic spikes in mature starburst cells was caused by the technique of whole-cell patch-clamp recording (e.g., due to intracellular dialysis), because under the same recording condition every starburst cell in the early developing retina was capable of generating TTX-sensitive somatic action potentials. It is also not likely that the staining of starburst cells by DAPI could have caused Na channels to disappear in the mature retina, because some of the spiking starburst cells recorded from the young rabbits were also stained by DAPI. On the other hand, we cannot exclude the possibility that, as the starburst cell matures, there might be a redistribution of Na channels toward distal dendrites where voltage-clamp is inadequate. It has been reported that dendritic varicosities become gradually aggregated in distal zones of starburst dendrites as the retina matures (35). Our results now can also explain the large variability in the spiking capability of starburst cells in our earlier reports at scientific meetings. In those preliminary studies, we only recorded from rabbits aged 2–3 weeks and older and, there-

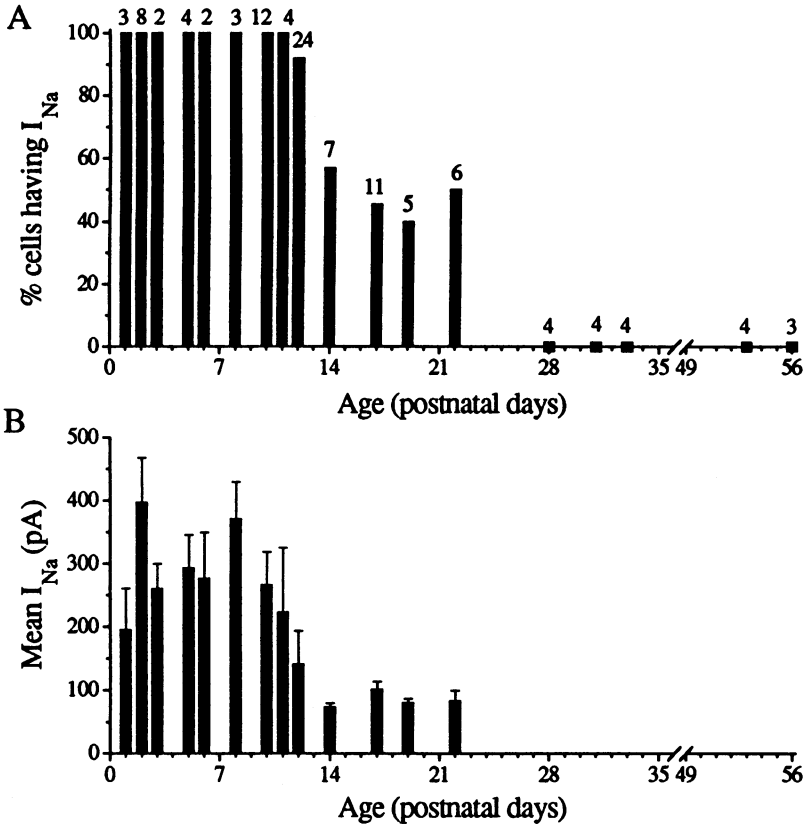

FIG. 5. Summary of Na currents from displaced starburst cells during postnatal development. (*A*) Percentage of starburst cells tested showing detectable (>15pA) Na currents under voltage-clamp (V_h = −70 mV). Data points shown on the abscissa at P28 and beyond indicate 0%. Numbers above the vertical bars indicate number of cells tested at each age. (*B*) Peak-amplitude of Na currents, averaged from cells that showed detectable (>15 pA, V_h = −70 mV) Na currents, as a function of age. Error bars indicate standard error.

fore, found only a small percentage of the starburst cells tested had TTX-sensitive Na currents and spikes. The amplitude of the spikes as well as Na currents in those preliminary studies was usually small.

In the mature retina, starburst cells have been proposed to participate in complex signal processing (such as directional selectivity), which may require semiisolated local dendritic subunits (23). Though the actual physiological role of starburst cells is currently unknown, our results support the notion that action potentials are not used to facilitate communication between different parts of the starburst cell in the mature rabbit retina. Moreover, the fact that starburst cells change specifically from spiking to nonspiking appears to indicate that action potential propagation in mature starburst cells is not only unnecessary but probably deleterious to whatever role these cells play.

It is interesting that in the developing retina starburst cells do generate action potentials. They then become nonspiking neurons after vision begins. Such an all-or-none transition from spiking to nonspiking is rather unusual and, as far as we know, has not been reported for other neurons during development. It is also in contrast to the ontogeny of ganglion cell excitability, which undergoes a gradual increase in spiking capability during early retinal development (44).

It seems possible that spiking in the starburst cell during a specific developmental period may have important functional consequences. Because the decline of the spiking capability in starburst cells coincides with the loss of the spatiotemporal coordination among spontaneously firing cells in the inner mammalian retina (43), one may speculate that starburst cells might function in the developing retina as a part of a network that generates the spontaneous activity crucial for neuronal connectivity. Such a role would be consistent with the symmetric morphology of starburst cells, the lateral organization and broad coverage of the cholinergic network in the IPL, and the neurochemistry of these cells (16–18, 23, 39, 45, 46). Recent observation that curare, a nicotinic receptor antagonist, blocks the correlated waves of spontaneous activity in many ganglion cells in neonatal ferret retina (52) also supports this view.

It is now becoming increasingly clear that regulation of intrinsic electrical properties of neurons, in addition to modification of synaptic efficacy and connectivity, plays an important role in neuronal development. It has also been suggested that activity-dependent changes in intrinsic electrical properties plays a role in maintaining stable properties of neurons in response to growth and changing inputs (47, 48). Alternative splicing of some Na channel genes has been shown to be developmentally regulated (49, 50). More interestingly, the expression of at least one form of Na channel in brain (rNaB3) seems to occur mainly in embryos and considerably less in adult (51), although little is known about the functional consequence of such a differential expression. Our results may provide an opportunity to understand the physiological correlation of Na channel gene expression during development. The regulation of Na channel expression in starburst cells may enable these cells to subserve one function in the developing retina and a very different one in the adult.

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