Dynamic microtubule-dependent interactions position homotypic neurones in regular monolayered arrays during retinal development

Lucia Galli-Resta1,*, Elena Novelli1 and Alessandro Viegi2
1Istituto di Neuroscienze CNR, Laboratorio di Neurofisiologia, Via G. Moruzzi 1, 56100 Pisa, Italy
2Scuola Normale Superiore, Via G. Moruzzi 1, 56100 Pisa, Italy
*Author for correspondence (e-mail: galli@in.pi.cnr.it)

SUMMARY

In the vertebrate retina cell layers support serial processing, while monolayered arrays of homotypic neurones tile each layer to allow parallel processing. How neurones form layers and arrays is still largely unknown. We show that monolayered retinal arrays are dynamic structures based on dendritic interactions between the array cells. The analysis of three developing retinal arrays shows that these become regular as a net of dendritic processes links neighbouring array cells. Molecular or pharmacological perturbations of microtubules within dendrites lead to a stereotyped and reversible disruption of array organization: array cells lose their regular spacing and the arrangement in a monolayer. This leads to a micro-mechanical explanation of how monolayers of regularly spaced ‘like-cells’ are formed.

Key words: Retina, Retinal mosaics, Patterning, Dendritic interactions, Microtubules, Rat

INTRODUCTION

Most regions of the central nervous system derive an enormous power of information processing from a typical modular architecture. The packing of neurones into sequential layers supports serial processing, while the positioning and the connectivity of neurones within each layer allow parallel processing. The retina well exemplifies these organizing principles. Each neuronal type is found within a specific layer and commonly cells of the same type orderly tile the retina (Masland, 1996; Wässle and Boycott, 1991). This organization can be so regular that arrays of like neurones in the retina are commonly referred to as mosaics (Cook and Chalupa, 2000; Wässle and Riemann, 1978).

Studies ranging from fish to primates have shown that the emergence of non-random arrays of like cells in the retina is an early step in development. Regular distributions of photoreceptors, amacrine and horizontal cells are observed before all the layers of the retina have been generated, and often before all the cells forming the mature arrays are born or have migrated to their layers (Bumsted et al., 1997; Bruhn and Cepko, 1996; Cook and Chalupa, 2000; Galli-Resta et al., 1997; Larison and Breimiller, 1990; Raymond et al., 1995; Scheibe et al., 1995; Wikler et al., 1997). The geometry of cell spacing within retinal arrays has provided important cues on the mechanisms controlling mosaic assembly, showing that many arrays derive their orderly organization from an exclusion rule: each cell is surrounded by a limited domain where other cells of that same type are never positioned (Cellerino et al., 2000; Cook and Chalupa, 2000; Galli-Resta, 1998; Galli-Resta, 2000; Galli-Resta et al., 1999).

Yet mechanisms that enforce a minimal spacing between homotypic neurones are not sufficient to account for mosaic assembly, as revealed by the three dimensional analysis of array development performed in recent studies (L. G.-R. and E. N., unpublished). By analysing the assembly of the cholinergic and the horizontal cell arrays, these studies unveiled a typical sequence of events in mosaic formation: neurones do not migrate to a precisely defined layer, but rather take position independently of one another in a broad band of retinal thickness. Gradually, a minimal spacing appears between like-neurones that remain located at different depths. Finally, like-neurones become positioned in a monolayer. Thus, cell spacing and the location of like-cells in a monolayer are the result of a continuous repositioning of cells.

Experimental manipulations of the cholinergic arrays in normal and transgenic models have shown that the regular spacing of these cells depends on short-range interactions that are restricted to homotypic cells, and are most probably contact mediated (Galli-Resta, 2000). Although this analysis has been performed systematically only for the cholinergic arrays, retinal arrays are likely to use common mechanisms for spacing their cells. This is suggested by the generality of the exclusion rule (Cellerino et al., 2000; Cook and Chalupa, 2000; Galli-Resta, 1998; Galli-Resta, 2000; Galli-Resta, 2001; Galli-Resta et al., 1999), which does not require anything but local interactions between homotypic cells and by the lack of correlation between cell spacing in different mosaics (which is an indicator of their mutually independent assembly) (Rockhill et al., 2000).

The cell dendritic tree stands as a natural candidate for supporting local cell interactions. Its highly dynamic nature,
and its remodelling in response to extracellular signals during development (Luo, 2000; Scott and Luo, 2001; Wong et al., 2000), make it an ideal tool for the cell to explore the surrounding environment and come into contact with neighbouring cells. These contacts could easily support local interactions between like-cells (Lohmann and Wong, 2001), and should necessarily do so if such interactions are contact mediated (Galli-Resta, 2000). Indeed, modelling studies show that like-cells moving to minimize dendritic overlap end up forming regular mosaics (Eglen et al., 2000).

To investigate the role of dendrites in array formation, we have searched for tools to manipulate selectively the processes of specific cell types. In particular, we intended to focus on the cholinergic and horizontal cells that form the earliest detectable arrays of the retina. We show here that the limited distribution of the mature forms of microtubule-associated protein 2 (MAP2) provides a means with which to focus selectively on the cholinergic cell dendrites. Reducing MAP2 levels by means of antisense oligonucleotides reversibly disrupts the cholinergic arrays, which lose the regular spacing of their cells, and their arrangement in monolayer, as shown here by statistical and computational analysis of cell positioning. The same stereotyped disruption of array organization was observed for the cholinergic and the horizontal cell arrays when performing more generalized molecular or pharmacological manipulations of the microtubules (MTs).

These results show that the spacing of homotypic cells and their arrangement in a monolayer depend on cell dendritic interactions. A micro-mechanical explanation is presented that accounts for the formation of regular monolayered arrays of homotypic neurones.

MATERIALS AND METHODS

Animal handling, immunocytochemical and histological procedures

Experiments were performed on Long Evan hooded rats in compliance to the national and the ARVO regulation on animal experimentation. Intraocular injections and optic nerve section in neonatal rats were performed under anaesthesia as described (Galli-Resta et al., 1997). BrdU pellets were applied subcutaneously on postnatal day 0 (P0; n=2), or P2 (n=2), or P4 (n=2), as described (Galli-Resta and Ensini, 1996). Eye collection, fixation, dissection, retina flat mounting, sectioning, single and double immunostaining were performed as described (Galli-Resta et al., 1997). Immunocytochemistry for cytoskeletal components was performed after simultaneous extraction and fixation, using a buffer described elsewhere (Schliwa et al., 1981). Cholinergic cells were detected by choline acetyltransferase (ChAT) or islet 1 expression. Horizontal cells were labelled with calbindin antibodies. Antibodies sources: ChAT (Chemicon), islet 1 (clone 4DS; gift of T. Jessel), BrdU, tau (Roche), calbindin, MAP2ab, MAP1, MAP5, β-tubulin (Sigma) and βFGF (Upstate).

Treatments

Antisense and sense oligonucleotides were obtained from Pharmacia and dissolved in phosphate-buffered saline (PBS). The MAP2 antisense oligonucleotides were RM21 (CTCGTCAGCCATCC- TTTAGATCTCT) and RM38 (GTACTGTCCTAATCACA- GTTGTA), described elsewhere (Caceres et al., 1992). The tau antisense oligo was RT11 (GGCTTTGAAGCAGCATTGG- GTTGTAA), described elsewhere (Caceres et al., 1992). The tau and dissolved in phosphate-buffered saline (PBS). The MAP2 were labelled with calbindin antibodies. Antibodies sources: ChA T elsewhere (Schliwa et al., 1981). Cholinergic cells were detected by

Immunoblotting and quantitative confocal immunofluorescence analysis of protein levels

Fifteen treated (T) and 15 control (C) retinas were taken 24 hours after oligo injection, pooled in samples containing three equivalent retinas each, and analysed by immunoblotting. Proteins were extracted with lysis buffer [Triton X-100 1%, glycerol 10%, TrisHCl 20 mM (pH 7.5)]. Samples from each analysed array were taken at regularly spaced locations going from the centre to the periphery of whole mounted retinas, along four (or more) regularly spaced retinal axes, using a Leica TCNS confocal microscope. Sampling regions were 400x400 µm². Typically, between 1/20 and 1/5 of each retina was sampled. The area of each retina was acquired with a CCD camera before and after reaction, and after data sampling to control for tissue shrinkage/compression. Sampled fields and retinal images were fed to an Image analyser (Imaging Ontario, Canada) to determine cell density, cell positioning and retinal area.

Histological controls

For each treatment, 30 µm cross sections from five control and five treated retinas were counterstained with the nuclear dye YOYO (Molecular Probes; 10-15 µg/ml). Immunocytochemical analysis of protein levels was performed by means of a custom-made program, as described (Galli-Resta et al., 1997). As each cell has one nearest neighbour, one Voronoi domain, but an average of five Delaunay segments, the Delaunay segments offer the most stable statistics. The analysis of Delaunay segments was limited to the cell coordinates in the retinal plane, but for a limited number of samples, where Delaunay segments were computed in three dimensions, and confirmed array disorganization after treatment (not shown). Cell scattering perpendicular to the layer (depth scatter) was quantified in confocal series through the array, assigning each cell to the frame where its nucleus first appeared.
MAP2ab (Sigma) 2 
Tween-20 (0.2%) in TBS for 2 hours, and incubated with mouse anti-
Blots were blocked with milk (4%) (BioRad non-fat dry milk) and
loaded with proteins from treated retinas alongside five control lanes.
Electrotransferred to nitrocellulose (1 hour, 100 V). Five lanes were
loaded with proteins from treated retinas alongside five control lanes.

5 minutes, loaded on a 3%-12.5% gradient SDS-PAGE and
curve. Proteins (30 µg) were added with sample buffer, boiled for

Assay kit (BioRad) using bovine serum albumin (BSA)-based standard
curves. Protein concentration of the samples were assessed with the BioRad protein

Electron microscopy (EM) is a powerful tool to study the ultrastructural

confocal analysis within the cholinergic cell processes. This analysis allowed to evaluate the oligo-mediated effects on the cell processes, and to detect differences in the effects on the two ChAT arrays in each single retina. For this analysis, sections of treated and normal eyes were mounted in the same block of inclusion medium, frozen, sectioned and processed together. Double labelling was performed using antibodies from different species to identify the array cells and their processes (ChAT for the cholinergic, calbindin for the horizontal cells), and the cytoskeletal protein of interest. For each protein, labelling intensity was averaged over 2000 1x1 µm² spots placed along the array cell processes in 100x confocal images acquired under fixed conditions in at least four different sections. A control for this method is provided by the results obtained for unaffected MAPs (see Results).

Statistical analysis
Plots and frequency histograms were produced by means of Origin
5.0 (Microcal). Statistical comparisons were performed using the two-tailed Kolmogorov-Smirnov (KS) test (Cook, 1996; Siegel and Castellan, 1988), as well as the bootstrap analysis (Efron and Tibshirani, 1991). The KS test was applied to compare each treated case to the average data obtained for the normal cases. The bootstrap was applied to compare the normal and the treated dataset, for each condition of treatment. This latter analysis has the advantages to avoid assumptions about the data distribution, and to take into account the variability within each dataset. In the bootstrap analysis, a treatment is scored as ineffective when the bootstrap estimate of the difference between the histograms obtained for treated and normal cases remains for all bins within the difference obtained comparing independent data sets from control cases. Effective treatments had a 50% or higher difference in at least one histogram bin with respect to what was obtained in the comparison of normal data sets. A more detailed explanation of the bootstrap method can be found elsewhere (Efron and Tibshirani, 1993). Bootstrap was performed by means of a custom-made program (Galli-Resta et al., 1999).

RESULTS

Mature retinal mosaics often consist of regularly spaced homotypic cells interconnected by a network of processes (Cook and Chalupa, 2000; Vaney, 1990). Observing the cholinergic (ChAT-positive) cells of the ganglion cell layer (GCL), while these are still partly irregularly positioned (L.

G.-R. and E. N., unpublished), we noticed that where these cells are orderly spaced, a network of ChAT-positive processes links neighbouring elements (Fig. 1A). Where gaps are observed in the net of ChAT-positive processes (Fig. 1B), the surrounding cells are usually irregularly spaced from one another (e.g. clusters of cells without minimal spacing indicated by arrows in Fig. 1B). Similar observations were made for the developing horizontal cell array (Fig. 1C,D). This suggests a correlation between the development of cell processes and array formation.

To investigate this issue, we searched for tools to alter selectively the processes of specific cell types. Neuronal processes have a structural core made of longitudinal bundles of microtubules (MTs), that derive their unusual length, stiffness and stability from specific neuronal microtubule associated proteins (MAPs) (reviewed by Heidemann, 1996). Previous studies have shown that the major neuronal MAPs have a restricted distribution in the developing retina (Tucker et al., 1989; Tucker and Matus, 1988). We confirmed these observations, and found that during the period of array development the retinal ganglion cells (RGCs) and the ChAT-positive cells are the only cells expressing MAP2ab, the mature form of MAP2. Thus, if RGCs are removed by optic nerve section at birth, the neurones forming the two ChAT-positive
arrays (Fig. 2A) remain the only retinal cells to express MAP2ab (Fig. 2B). In these conditions, manipulations altering the levels of MAP2 could be a way to affect selectively the dendrites of the ChAT-positive cells.

Phosphorothioate (PP) antisense oligonucleotides to MAP2, with sequences known to reduce MAP2 expression in cultured neurones (Caceres et al., 1992), were injected intracocularly in neonatal rats on P0, after optic nerve section. FITC-conjugated versions of these sequences showed that oligos penetrate through the entire retinal thickness in four out of five tested retinas (Fig. 2C), while in a fifth case the penetration was limited to the GCL and the innermost INL (Fig. 2D). Twenty-four hours after oligo injection, MAP2ab levels were significantly reduced in the treated retinas (Fig. 2E,F), as quantified by immunoblotting (50±10% reduction, Fig. 2G). When assessed by means of quantitative confocal immunofluorescence, MAP2 level reduction appeared even stronger in the cholinergic cell processes (80% reduction), where the levels of MAP1A and MAP5, analysed as a control, remained unaffected (Fig. 2H).

Treatment with oligo antisense to MAP2 did not affect the retina in obvious general ways: 24 hours after oligo injection, treated (T) and control (C) retinas were indistinguishable in terms of stratification pattern (Fig. 2I, J), thickness (T, 184±19 μm, n=5; C, 187±16 μm, n=5), numbers of pyknotic cells (T, 7.5±3 per mm²×10³, n=5; C, 8±3 per mm²×10³, n=5), and number of mitotic cells (T, 19.2±7 per mm²×10³, n=5; C, 18.4±5 per mm²×10³, n=5). Yet, the ChAT-positive arrays were completely disrupted 24 hours after treatment with oligos antisense to MAP2. Arrays of regularly spaced cells (Fig. 3A) turned into disordered distributions of neurones, where clusters of cells without any minimal spacing alternated to regions where very few ChAT-positive cells could be seen (Fig. 3B). Furthermore, the cells of either ChAT-positive array become scattered at different retinal depths (e.g. arrows in Fig. 2E), losing their mono-layered organization. A few days later (P7), when MAP2 levels have recovered (Fig. 2G), the cholinergic arrays of the treated retinas (n=9) are indistinguishable from normal (Fig. 3C). This dramatic, reversible alteration of the uniform sampling of the retina that retinal mosaics normally ensure before P12 (Galli-Resta and Novelli, 2000) can be quantified by the oscillation in the density of the ChAT-positive cells. When analysed in clusters of adjacent 125×125 μm² fields, cell density varies very little in normal retinas, although it varies significantly 24 hours after treatment with MAP2 antisense oligo. This is illustrated by the examples in Fig. 3D, where the grey band represents the maximum density variation observed in either ChAT-positive arrays of a normal P1 retina,
Fig. 3. Intraocular injections of MAP2 antisense reversibly disrupt the ChAT-positive arrays. (A–C) The regular intercellular spacing of the ChAT-positive arrays observed before treatment (A, P0) is disrupted 24 hours after treatment with oligo antisense to MAP2 (B, P1). At P7, when MAP2 has recovered (see Fig. 2G), the ChAT-positive cells are reorganized into regular arrays (C). Scale bars: 20 μm. (D) An example of the density oscillation of the cholinergic neurones across the retina 24 hours after treatment with MAP2 antisense oligonucleotide at P1. As a comparison, the grey band represents the interval between the maximal and minimal density values observed in either cholinergic arrays of a normal littermate retina. Notice that cell density in either ChAT-positive arrays does not normally vary with eccentricity at this age, as it has been shown to occur until P12 (Galli-Resta and Novelli, 2000). Density is measured in clusters of adjacent 125×125 μm² sampling fields taken at four different retinal eccentricities (eccentricity is the distance from the centre of the retina; 0%=centre). Filled symbols refer to the GCL ChAT-positive cells of the treated retina, open symbols to the treated INL ChAT-positive cells. (E) No difference in the density variation of ChAT-positive cells is observed between treated and normal littermates on P7, indicating complete recovery. Symbols as in D. The significant growth of the retina between P1 and P7 (McCall et al., 1987) explains the decrease in the average density of ChAT-positive cells observed between P1 (E) and P7 (D). (F) Treatment with MAP2 antisense affects the INL and GCL ChAT-positive arrays independently of one another, as shown by the ratio between the cell density in these two arrays at different retinal locations. The ratio between the density of INL ChAT-positive cells and the density of the GCL ChAT-positive cells is plotted as a function of eccentricity for the same treated retina shown in D. As a comparison, the grey band comprises the values obtained for this ratio in the normal retina shown in D. Although the INL/GCL density ratio variance is limited for the normal retinas, it varies considerably from field to field in the treated retinas, showing that there is no correlation between the effects on density observed in the GCL and in the INL ChAT-positive array of the treated retinas. (G) No significant difference in the number of cholinergic cells was observed between treated (T) and normal control (C) retinas at the time of array disassembly (P1) or after recovery (P7). Open triangles, INL ChAT-positive cells; black triangles, GCL ChAT-positive cells. The cholinergic cells were labelled with two different markers (ChAT and Islet 1), which produced indistinguishable results.

while the symbols represent the density values observed in adjacent fields of the GCL (filled symbols), and of the INL ChAT-positive array (open symbols) of a treated retina from a littermate animal.

When analysed in clusters of 3×3 adjacent 125×125 μm² fields (see Materials and Methods) across 10 normal retinas, cell density varies of an average factor of 1.12±0.13, with a maximum of 1.6-fold variation, in either ChAT-positive arrays. When 10 retinas are similarly analysed 24 hours after treatment with MAP2 antisense oligo, density ratio in clusters of adjacent fields can rise by up to a factor of 5, with an average of 1.78±0.8 for the GCL ChAT-positive array, and of 1.58±0.6 in the INL ChAT-positive array. Both values are significantly different from normal (t-test, P<0.001 for both arrays). When MAP2 levels are restored (Fig. 2G), array cells recover a constant density across the retina (Fig. 3E). This density is lower than on P1 (Fig. 3D), for the effects of retinal growth (McCall et al., 1987).

No significant difference in the number of cholinergic cells was observed between treated and normal retinas at the time of array disassembly (P1) or after recovery (P7), independent of the marker used to identify the cholinergic cells (pChAT, Islet 1; Fig. 3G). Furthermore, BrdU incorporation experiments performed after treatment did not show the entry of newly generated cells in the ChAT-positive arrays (no BrdU-labelled cells in 3000 ChAT-positive cells analysed in six retinas treated with antisense oligos to MAP2 on P0). Thus, array disruption and reorganization must be due to the spatial rearrangement of the array cells.

To investigate the effects of the treatment on the geometrical organization of the ChAT-positive arrays, we analysed the distribution of the Delaunay segments, which quantify how cells are spaced from their surrounding neighbours, and the distribution of cells as a function of retinal depth (Fig. 4). The characteristics and the variability of these distributions among normal retinas are shown by the grey curves in Fig. 4. The normal distribution of Delaunay segments shows that the spacing between each array cell and its surrounding neighbours is regularly distributed in a bell-shaped curve, where distances below 12 μm or above 50 μm are hardly ever observed. Twenty-four hours after treatment with MAP2 antisense, the distribution of Delaunay segments was significantly altered, spanning distances outside the normal range, as a consequence of clustered and sparse cholinergic cells (as shown by the examples in Fig. 4B). The distribution of array cells as a function of retinal depth normally displays a sharp peak (grey curve in Fig. 4F-H), as retinal arrays form monolayers. This distribution becomes broader and shorter than normal after treatment with oligo antisense to MAP2, as the treatment scatters cells at different retinal depths (as shown by the examples in Fig. 4F). A summary of the results is in Table 1.

Different experiments confirm the specificity of the
Antisense oligos to MAPs disrupt the orderly spacing of the ChAT-positive cells and scatter these cells at different retinal depths. (A) Delaunay segments link array cells (dots) with their surrounding neighbors. (B) Examples of the altered distributions of Delaunay segments (DS) associated to the GCL ChAT-positive array 24 hours after treatment with MAP2 phosphorothioated antisense oligos (black circles, 25 μM PPRM21; black squares, 25 μM PPRM38), or with MAP2 antisense with phosphorothioate links only at the 3’ and 5’ ends (continuous line indicates 50 μM ppRM21). The DS associated with normal and vehicle injected cases are shown as grey lines. Each curve is the average DS distribution of a single retina: as shown, the extent of the oligo effect varies significantly between treated retinas, but it always includes DS lengths outside the normal range. Each treated DS plot is significantly different from the normal DS histogram (KS test; \( P<0.0001 \)). The difference between the treated and the normal cases was confirmed by the bootstrap method, which allows the comparison of datasets, taking into consideration their internal variability (see Materials and Methods). (C) Examples showing that the DS distribution of the GCL ChAT-positive array did not vary significantly with: (1) the phosphorothioate sense sequences complementary to the antisense oligos used (black circles, 25 μM RM21 sense; black squares, 25 μM RM11 sense); (2) blocking antibodies to βFGF (white circles, 10 μg/ml), used to simulate potential non-specific effects of PP oligos. Normal DS distributions are grey lines. Each treated DS plot was not significantly different from the normal DS histogram (KS test \( P<0.01 \)). This result was confirmed by bootstrap analysis (see Materials and Methods). (D) Examples of the alteration of the DS distribution after treatment with a phosphorothioate antisense to tau (PPRT11: upward facing triangles, 12 μM; downward facing triangles, 25 μM). Normal DS are the grey curves. Each treated DS plot is significantly different from the normal DS histogram (KS test; \( P<0.0001 \)). The difference between the treated and the normal cases was confirmed by the bootstrap method, which allows the comparison of datasets, taking into consideration their internal variability (see Materials and Methods). (E) Schematic representation of cell scatter in depth. Dots represent cells, the line an arbitrary reference depth. (F-H) Examples of the scatter of the GCL ChAT-positive cells at different retinal depths after treatment with MAP2 antisense (F), control treatments (G) and tau antisense (H). Each curve represents data from a single retina (see above). Symbols are as in B-D. The treated cases are illustrated upside-down to facilitate comparison with the normal controls (grey curve). The statistical significance of the effects was assessed by means of the bootstrap method and the K-S test \( P<0.001 \). Between 5 and 20% of each retina was sampled for this analysis.

Antisense effects, as shown by the examples of Fig. 4 and by the summary in Table 1. Array disruption was observed with two different phosphorothioated (PP) MAP2 antisense oligos (PPRM21, \( n=18 \); PPRM38, \( n=7 \)), each used at two different concentrations (12.5 μM and 25 μM). Furthermore, oligos with the same sequences, phosphorothioated-modified only at the 3’ and 5’ ends (ppRM21 50 μM, \( n=5 \)), proved effective in disrupting array organization, a test speaking against potential non-specific effects of phosphorothioation. On the contrary, arrays were not affected when injecting the PP sense oligos (sPPRM21 \( n=4 \), 25 μM), or blocking antibodies to βFGF (1 μg/ml; 10 μg/ml; 40 μg/ml; \( n=3 \) each), used to simulate the potential non-specific binding of this factor by PP oligos (Guvakova et al., 1995; Retaux et al., 1996). Altogether, these results indicate that array disruption is a consequence of reduced MAP2 levels.

Oligo injections performed at different ages showed that MAP2 antisense oligonucleotides disrupt the organization of the cholinergic arrays on P0, P2 or P4, but not on P6, or P15 (Table 2).

Treatment with oligos antisense to MAP2 affected the two cholinergic arrays independently of one another: Cell density varied independently in the two arrays (Fig. 3F), and we found five cases out of 30 tested where only the GCL array was affected (Table 1), as for each of these cases both the DS and the depth scatter distributions for the INL array were indistinguishable from normal (KS test \( P<0.01 \)). Furthermore, array disruption occurred independently of other cells
When array disruption occurs, processes that link array cells are still observed and contain microtubules throughout. Double labelling for ChAT (green) and β-tubulin (red) of a region of the GCL cholinergic array after treatment with MAP2 antisense. (A) Stack of confocal sections. (B-D) Confocal section through A (0.5 μm), showing the double labelling (B) and ChAT (C) and β-tubulin (D) immunoreactivity. Although the array cells are irregularly spaced, the ChAT-positive processes linking array cells contain microtubule throughout, as revealed by β-tubulin immunoreactivity (e.g. the processes linking cells 2-3 or 3-5).

### Table 1. Effects of different treatments on the ChAT arrays

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DS alteration</th>
<th>Depth scatter alteration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without MAP2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPRM21 12 μM</td>
<td>7/8</td>
<td>5/5</td>
</tr>
<tr>
<td>PPRM38 12 μM</td>
<td>3/3</td>
<td>3/2</td>
</tr>
<tr>
<td>ppRM21 50 μM</td>
<td>5/5</td>
<td>3/3</td>
</tr>
<tr>
<td>Total</td>
<td>29/30</td>
<td>18/18</td>
</tr>
<tr>
<td>Without tau</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPRRT11 12 μM</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td>25 μM</td>
<td>4/4</td>
<td>3/3</td>
</tr>
<tr>
<td>Total</td>
<td>7/7</td>
<td>6/6</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sPPRM21 25 μM</td>
<td>0/4</td>
<td>0/2</td>
</tr>
<tr>
<td>sPPRT11 25 μM</td>
<td>0/4</td>
<td>0/2</td>
</tr>
<tr>
<td>Vehicle 1:1</td>
<td>0/5</td>
<td>0/2</td>
</tr>
<tr>
<td>Anti FGFβ 1 μg/ml</td>
<td>0/3</td>
<td>0/2</td>
</tr>
<tr>
<td>10 μg/ml</td>
<td>0/3</td>
<td>0/2</td>
</tr>
<tr>
<td>40 μg/ml</td>
<td>0/3</td>
<td>0/2</td>
</tr>
<tr>
<td>DMSO 1:250</td>
<td>0/25</td>
<td>0/14</td>
</tr>
<tr>
<td>Total</td>
<td>0/25</td>
<td>0/14</td>
</tr>
</tbody>
</table>

For each treatment, the table indicates the number of cases where an effect was observed over the total number of retinas analysed. Each agent was injected intraocularly at P0, and the effects analysed 24 hours later. Thirty to 50% of the animals analysed in each case had the optic nerve sectioned at birth.

In normal cases 10-15% of the DS are <15 μm and 90-95% are within 50 μm (see Fig 4B-D). We considered treatments as effective if they led to DS distributions where either more than 20% of the DS were below 15 μm or less than 85% were below 50 μm. Depth scatter was considered to be affected when s.d. > 3 μm, because in normal cases s.d. < 1.5 μm. In normal cases 10-15% of the DS are <15 μm (see Fig 4F-H). For the control cases, the DS and the depth scatter distributions were indistinguishable from normal (KS test P<0.01 and bootstrap analysis).

### Table 2. Effects of oligo antisense to MAP2 at different ages

<table>
<thead>
<tr>
<th>Age</th>
<th>DS segment alteration</th>
<th>Depth scatter alteration</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0</td>
<td>29/30</td>
<td>18/18</td>
</tr>
<tr>
<td>P2</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td>P4</td>
<td>8/8</td>
<td>4/4</td>
</tr>
<tr>
<td>P6</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>P15</td>
<td>0/5</td>
<td>0/5</td>
</tr>
</tbody>
</table>

The data for P2, P4, P6, P15 were obtained injecting RM21 25 μM on the age indicated, and analysing the ChAT arrays 24 hours later. Details for the P0 cases are in Table 1. Half of the P4 cases analysed had the optic nerve sectioned at birth to eliminate RGCs.

In the unaffected cases the DS and the depth scatter distributions were indistinguishable from normal (KS test P<0.05 and bootstrap analysis).

expressing MAP2ab, because it was observed in normal retinas and in retinas where RGCs had died as a consequence of optic nerve section at birth (Table 1). These results indicate that the treatment must directly affect the elements of the array to lead to array disruption, no matter what the effects are on other cells that express MAP2.

Reducing MAP2 levels leads to array disruption, but the cell processes linking neighbouring ChAT-positive cells can still be observed, and β-tubulin immunoreactivity performed after monomeric tubulin extraction (see Materials and Methods), reveals that these processes contain bundles of microtubules (MTs) throughout (Fig. 5A-D). This indicates that process disruption is not necessary to induce array disassembly, and that more subtle alterations brought about by the treatment are sufficient to induce array disruption. As MAP2 is associated with the microtubules, this suggests that microtubule alterations are the cause of mosaic disassembly.

To test this issue further we tried different perturbations affecting MTs. None of these was restricted to a specific cell type. However, we had reasons to expect that each array would be affected only if MTs were altered within the cells forming the array, independently of the effects on other cell types. This is based on the notion that arrays are assembled and maintained by interactions restricted to the array cells (Galli-Resta, 2000; Rockhill et al., 2000), and is verified by the above experiments with MAP2 antisense oligos, showing that either cholinergic array is affected only when the treatment affects directly its cells. As a direct control for this hypothesis, however, in each of the following experiments, we tested whether the alterations of cell density in the different arrays are independent of one another.

In the next set of experiments we used oligos antisense to tau, a second neuronal MAP found in the processes of the cholinergic and the horizontal cells, as well as in the RGCs,
and in a few other cells of the INL (Tucker and Matus, 1988). Intraocular injections on P0 of 12.5 μM (n=3) or 25 μM (n=4) of the phosphorothioated RT11 oligo led to the disruption of the cholinergic arrays much in the same way as MAP2 level reduction did. Cell clusters and regions of sparse cells were observed, and array cells lost their arrangement in monolayer (examples in Fig. 4D,H; see summary in Table 1). Similarly, the two arrays recovered their organization when tau levels recovered (not shown). No effect was observed when using the PP sense oligos (sPPRT11 n=4, 25 μM; Table 1). Thus, as MAP2 level reduction disrupts the cholinergic arrays, so does the reduction of tau, a different MAP normally found in the ChAT-positive processes.

Tau is also found in the processes of the horizontal cells, that form a regular array from P6 (L. G.-R. and E. N., unpublished). Injections of tau antisense at P6, reversibly disrupted the horizontal cell array (Fig. 6A,B) in the same stereotyped way observed for the disruption of cholinergic arrays: cell lost their regular tiling of the retina and became scattered out of their monolayer (see examples in Fig. 6C,D). In the eight retinas analysed 24 hours after injection of oligo RT11 antisense to tau (25 μM) the horizontal cells had less than 86% of their DS within the interval between 15 and 60 μm, an interval that normally comprises 92-95% of the DS for the horizontal cells in the normal retina (e.g. grey curves in Fig. 6C). Similarly, the depth scatter associated to the horizontal cells in these treated retinas had a standard deviation 1.4-1.7 times that observed in the normal retinas. Treatment with antisense to tau at P6 did not affect the cholinergic cell arrays (n=4; KS test P<0.01 for the DS and the depth scatter histograms of both ChAT-positive arrays), confirming that the effects on different arrays were not correlated. These results suggest that retinal arrays are build by independent, yet similar mechanisms, based on the processes of the array cells, and specifically on the properties that the processes derive from their mature MT compartment.

In a final set of experiments we used different pharmacological agents affecting MTs. Two of these, demecolchid and nocodazol, depolymerize MTs (Taylor, 1965; Vasquez et al., 1997), while the third, paclitaxel, stabilizes MTs against depolymerization, but makes them more flexible (Felgner et al., 1996). As these drugs are likely to affect MTs in all retinal cells, we used minimal concentrations that affect MTs, as assessed by previous studies (Ahmad et al., 2000; Matthews et al., 1982). Intraocular injections of demecolchid (10 μM, n=12), or nocodazole (5 μg/ml, n=12), or paclitaxel (5 μM, n=12) were performed on P0, and the retinas analysed 0.5, 2, 4 and 24 hours later. Confirming previous studies (Matthews et al., 1982), an increase in the number of mitotic cells induced by demecolchid spindle blockade was the only massive histological modifications of the retina observed with these drug dosages: 4 hours after treatment, the pattern of stratification (Fig. 7A), retinal thickness (T, 185±24 μm, n=5; C, 187±16 μm, n=5), and the number of pyknotic cells (T, 7.4±5 per mm³×10³, n=5; C, 8±3 per mm³×10³ n=5) were indistinguishable from normal. By contrast, the ChAT-positive arrays were altered within 30 minutes of drug administration (not shown), and showed more marked effects 2 and 4 hours later. The effects consisted in the same stereotyped disruption observed after MAP2 or tau level reduction (see examples in Fig. 7B,C). Cell clusters alternated to sparse cells, and array cells become scattered on different retinal depths (Table 3). Examples of the effects of demecholchid or paclitaxel on the DS of the GCL ChAT-positive array are shown in Fig. 7D. In general, these effects were less dramatic than when using MAP antisense, possibly for the mild dose of MT drugs used. Twenty-four hours after treatment the cholinergic arrays recovered their organization (examples in Fig. 7D; Table 3). Similar results were obtained on P0 for the INL ChAT-positive array, and on P6 for the horizontal cell array (Table 3). No correlation was observed in the density alteration induced by the treatment on the different arrays (not shown).

**DISCUSSION**

We have observed that as the cholinergic and the horizontal cell mosaics of the retina are ordered, a continuous network of processes links neighbouring array cells. Experimental manipulations that affect MTs within the processes disrupt the spatial patterning of these retinal mosaics. Within days or even hours, however, arrays are able to reorganize their orderly structure.

Five different molecular or pharmacological perturbations of MTs (oligos antisense to MAP2 or tau, depolymerization by demecolchid or nocodazole, paclitaxel treatment) lead to the same stereotyped effects: arrays turn into an alternation of cell clusters without any minimal spacing and zones of very sparse array cells. Furthermore, array cells become scattered at different retinal depths.

Albeit often more drastic, this disorganization is reminiscent...
of the initial disorder found among array cells early in development, when neither a monolayered arrangement nor a regular intercellular spacing characterizes the positioning of homotypic cells (L. G.-R. and E. N., unpublished). This suggests that the same mechanisms that assemble the arrays, allow them to recover from disruption. Indeed, the same experimental manipulations caused the disruption of the ChA T-positive arrays when these were already regular (injections on P2-P4), and prevented their complete development at earlier ages (injection on P0). In all cases arrays recover, suggesting that as cells are left to their normal resources, they re-activate the process of array assembly and maintenance. This plasticity may not characterize retinal arrays throughout life, as either the loss of this dynamic behaviour, or a reduced dependence on single MAPs are likely to account for the lack of any effects on the ChAT-positive arrays observed when MAP2 antisense are administered after P6. Interestingly, this time window for disruption (≤P4) corresponds in normal development to the period when the ChA T-positive cells are likely to move within their layer in order to accommodate new cells in the ChAT-positive arrays (Galli-Resta et al., 1997).

Array disruption and subsequent reorganization occur without changes in the number of cholinergic cells, as no difference in the number of array cells is observed between treated and normal cases of the same ages, no matter how the cells are labelled. Furthermore, array disruption and reorganization occur without the genesis of new array cells, as shown by the lack of BrdU-labelled cells within the arrays. Similar observations were made for the horizontal cells (not shown). Thus, cell displacement must account for both the loss of orderly cell spacing after treatments, as well as for the recovery of array organization that ensues. The same mechanism of cell movement within the array has already been hypothesized as a basic process in normal array development, because the analysis of X-inactivation transgenic mice reveals that cells forming retinal mosaics undergo tangential migration (Galli-Resta et al., 1997; Reese et al., 1999; Reese et al., 1995).

The fast array disruption following perturbations affecting MTs, and the subsequent recovery, indicate that retinal arrays are highly dynamic structures based on the MT component found within their cells. Agents that affect MTs within all retinal cells, as well as treatments that target MTs only within the array cells lead to the same stereotyped, reversible disruption of the retinal arrays analysed. This suggests that MT alteration within the array cells cause array disruption, no

**Table 3. Effects of MT drugs on the cholinergic and horizontal cell arrays**

<table>
<thead>
<tr>
<th>Time after treatment</th>
<th>Demecolchid (10 μM)</th>
<th>Nocodazole (5 μg/ml)</th>
<th>Paclitaxel (5 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ChAT-positive arrays*</td>
<td>Horizontal cells†</td>
<td>ChAT-positive arrays*</td>
</tr>
<tr>
<td>30 minutes</td>
<td>2/2</td>
<td>n.d</td>
<td>n.d</td>
</tr>
<tr>
<td>2 hours</td>
<td>2/2</td>
<td>2/2</td>
<td>2/2</td>
</tr>
<tr>
<td>4 hours</td>
<td>2/2</td>
<td>2/2</td>
<td>2/2</td>
</tr>
<tr>
<td>24 hours</td>
<td>0/4</td>
<td>n.d</td>
<td>0/4</td>
</tr>
</tbody>
</table>

The data refer to both the DS and the depth scatter, as the same figures were obtained for both. The criteria used to classify the ChAT arrays as affected are in Table 1. Criteria for the horizontal cells are indicated in the text.

n.d. = not determined.

In the unaffected cases, the DS and the depth scatter distributions were indistinguishable from normal (KS test P<0.05 and bootstrap analysis).

*Injection at P0, same figures for the GCL and the INL array.

†Injection at P6.
matter what the perturbation effects are on other cells. Using antisense oligos, we have found that array disruption is induced by a reduction in MAP2 levels, as several controls confirmed the specificity of oligo effects. At the ages analysed, MAP2ab is expressed by the ChAT-positive and the RGCs cells (Tucker et al., 1989; Tucker and Matus, 1988), and after reducing MAP2 levels, the ChAT-positive arrays are disrupted independently of the presence of the RGCs. Thus, array disruption must be caused by a direct effect of the treatment on the ChAT-positive cells. Furthermore, following MAP2 level reduction, each ChAT-positive array is affected independently of the other: cell density varies independently in the two cholineric arrays, and in some cases only one of the two array is affected. These extreme cases most likely reflect an occasional reduced penetration of the oligos, and yet represent a direct confirmation that each array is affected independently of the effects on other cells.

In general, the hypothesis that each array reacts to MT perturbations occurring within its cells has been confirmed in several ways, from the presence of arrays unaffected by the treatment (tau oligos at P6 only affects the horizontal cells; in some cases MAP2 oligos only affect the GCL ChAT-positive array), to the lack of correlation in the effects observed on the different arrays. This cell-type autonomy of array disruption reflects the mutual independence of array assembly; several previous studies have shown that different retinal arrays are spatially uncorrelated (Rockhill et al., 2000) and are based on a spacing rule that requires only interactions restricted to homotypic cells (Cellerino et al., 2000; Galli-Resta, 1998; Galli-Resta, 2000; Galli-Resta et al., 1999).

The experiments presented here indicate that the processes that array cells normally develop are necessary for cellular interactions allowing array formation and maintenance. These processes correspond to the cell dendritic tree, albeit this identity is somewhat unconventional in the case of amacrine cells that lack axons. Several experiments have shown the highly dynamic nature of the neuronal dendritic tree in developing cells (Gao et al., 1999; Ghosh, 2000; Luo, 2000; Scott and Luo, 2001; Wong et al., 2000), which may explain why perturbing the dendritic trees of the array cells results in such rapid effects on the cell array. Remodelling of dendrites has been shown to be modulated by cellular interactions, which can be limited to local interactions restricted to homotypic cells, as found in many cells, from the *Drosophila* md neurones (Gao et al., 2000) to the vertebrate retinal ganglion cells (Lohmann and Wong, 2001; Perry and Linden, 1982; Wassle et al., 1981; Wong et al., 2000; Wong and Wong, 2001). In all these cases, neurones appear to be able to limit the extension of the dendritic tree of neighbouring homotypic cells. If each array cell could prevent like-cells from taking position within the central region of its dendritic tree, this exclusion mechanism would suffice to reproduce cell spacing within the retinal array (Eglen et al., 2000; Galli-Resta, 2000).

Dendritic interactions are also responsible for the arrangement of mosaic cells in monolayers: cell scatter at different retinal depths invariably follows MT perturbation and arrays reform monolayers as they recover a regular intercellular spacing. But why should like-cells make a regular monolayered array? The role played by MTs in dendritic interactions may be the key to this answer, but here we face the greatest limitations of the present experiments. We can simply attempt to infer an explanation based on a process of elimination.

We have shown that affecting MTs in several different ways leads to the same reversible stereotyped effects. This suggests that there is a minimal set of MT alterations that all the treatments induce and that suffices to cause array disruption. Useful indications come from the observations of ChAT-positive arrays after reducing MAP2 levels – these are the most specific experiments, given the limited distribution of this MAP in the retina. After treatment with MAP2 antisense, the ChAT-positive arrays are disrupted but processes linking neighbouring cells can still be observed and contain bundles of MTs throughout. Thus, array disruption does not require generalized effects on MTs throughout the retina, nor the loss of the processes linking array cells or the loss of the MT bundles within them. It could be reasoned that all the perturbations affect intracellular transport, and thus disrupt interactions based on cell dendrites. There are indications, however, that this is not a likely consequence of all the perturbations performed. When the cell dendrites are still detected, as in the case of MAP2 antisense (Figs 2, 5) or tau (not shown) or paclitaxel (Fig. 7), these appear to contain ChAT immunoreactivity, as well as VChAT and synaptotagmin (not shown) throughout. These antigens are known to be associated to vesicles, and should not be found along dendrites, if intracellular transport is hampered. Furthermore, MAP2 and tau are not involved in transport along MTs (Kamal and Goldstein, 2000), so reducing the levels of these MAPs should not block transport. Finally, paclitaxel is known to preserve transport along MTs (Sloboda, 1992), and yet it causes array disruption, indicating that major alterations of intracellular transport are unlikely to be the outcome of all the treatments tested.

A common effect of all the manipulations performed is a weakening of the MTs that represent the rigid component of the cytoskeleton within neuronal processes: Demecolchid and nocodazole depolymerize MTs (Taylor, 1965; Vasquez et al., 1997). Paclitaxel makes MTs more flexible (Felgner et al., 1996). Without MAP2 or tau, MTs are shorter, less bundled and more flexible than normal (Felgner et al., 1997; Heidemann, 1996). Thus, all the MT perturbations we performed are bound to reduce the mechanical stiffness of the cell dendrites, much like an arm is weaker when its bones are shortened, thinned or made more flexible.

All the results reported can be accounted for by viewing each array as a net of mechanically interconnected like-cells. In this model, each cell is linked by its processes to its surrounding like neighbours. The rigid component of the processes is based on MTs, and counteracts an elastic component keeping the net together. A monolayer of orderly spaced cells is the equilibrium configuration of this net: as all the cells of the net are alike, they are regularly spaced at equilibrium and are arranged in the minimal energy configuration of a smooth one-cell-thick surface. If the stiff spacers between cells are haphazardly destabilized (MAP inhibition, MT depolymerization, more flexible MTs), the net wrinkles and loses its monolayered appearance: cell clusters form where microtubules are weakened first, consequently reducing the density of array cells somewhere else if the net remains continuous, and more so if it breaks. Being separate nets, the arrays behave independently one of the other. Finally, as MTs
recover their normal status within the cell processes, the nets return to their equilibrium configuration, i.e. the arrays recover their normal architecture.

This model is not intended to under-rate the complex molecular interactions that array formation no doubt requires. Rather, we suggest that the biochemical and cytological responses to mechanical forces are orchestrated at the cellular level so that array neurones behave as components of a mechanical net. Seminal studies by Heidemann, Buxbaum and collaborators (reviewed by Heidemann, 1996; Heidemann et al., 1999) have shown that neuronal processes react to mechanical forces like visco-elastic fluids. In particular, processes develop an internal tension, grow in response to an applied tension when this overcomes a threshold (Heidemann and Buxbaum, 1990; Zheng et al., 1991) and pull the cell body, which can be repositioned by mechanical forces (Lamoureux et al., 1989). This mechanical behaviour of neurones well fits with the model we propose for retinal mosaic assembly, provided that homotypic neurones have similar mechanical properties and form a continuous mechanical net.

The present experiments set only some firm points, showing that a cell layer of regularly spaced homotypic neurones is assembled by dendritic interactions that are perturbed as the MT component of the processes is altered. The role played by MTs is probably due to their mechanical properties. How the net of processes linking neighbouring homotypic cells is formed is still obscure, as are the possible molecular/structural bases of the net mechanical continuity. At the present moment a mechanical hypothesis of array formation simply provides a powerful model framework accounting, with a few general hypotheses, for all the experimental evidence acquired so far on retinal mosaic assembly. Testing how correct, and how general, this model and its predictions are will require many future experiments.

This work was supported by the CNR and the Telethon Foundation. We thank G. Resta for computer programming, and A. Fiorentini and M. C. Cenni for helpful comments on the manuscript.

REFERENCES


appears to be the primary factor producing the ganglion cell density gradient in the rat. *Neurosci. Lett.* 79, 78-84.


