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Immunocytochemical analysis of retinal neurons under electrical stimulation

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Abstract

To function successfully, a retinal prosthesis needs to provide effective stimulation in a safe manner. To date, most studies have been dedicated to assessing proper stimulation parameters, for example, determining stimulus threshold. Few studies have looked at the effects of prolonged stimulation on retinal morphology. One previous study did show gross morphological changes in the rat retina due to mechanical pressure, with and without electrical stimulation (Colodetti et al., 2007). Here, we used immunocytochemistry to investigate the effects of the same experimental conditions on neuronal structure in finer detail. For this purpose, we first defined four experimental groups. In Group 1, the stimulating electrode was near but did not contact the retina, and we did not apply current pulses. In Group 2, the electrode also did not contact the retina, but we applied current pulses of 0.09 $\mu\text{C}/\text{phase}$. In Group 3, the stimulating electrode directly contacted the retina, but we did not apply current pulses. In Group 4, the stimulating electrode directly contacted the retina, and we applied current pulses of 0.09 $\mu\text{C}/\text{phase}$. We found neural damage only in the outer retina, including a disturbance of synaptic vesicle proteins in the photoreceptor terminals and a remodeling of horizontal and rod bipolar cells' processes. These results show that, although gross morphological changes are mainly concentrated around the area of electrode contact, immunocytochemistry can reveal changes in adjacent areas as well.

Keywords

Mechanical pressure; Electrical Stimulation; Retina; Morphology; Immunoreactivity

1. Introduction

Electrical stimulation of the central nervous system, though unnatural, has been found to be an effective way of causing neuronal excitation. Neuroprostheses employing electrical stimulation strive to restore lost functionality by causing adequate and focal stimulation of target neurons (Javaheri et al., 2006; Loewenstein et al., 2004; Veraart et al., 2004; Weiland

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et al., 2005; Zrenner, 2002). Retinal prostheses specifically aim to create visual percepts by stimulating undamaged neurons of the inner retina in the case of diseases such as retinitis pigmentosa (RP) or age-related macular degeneration (AMD). Both are outer retinal diseases that primarily affect the photoreceptors, leading to partial or complete blindness (Humayun et al., 1999; Santos et al., 1997). For any such prosthesis to be successful, it not only has to provide adequate stimulation to cause excitation of the target neurons, but it also has to do so without causing damage to either the implant/electrodes or the surrounding biological environment.

Extensive electrophysiological studies have been conducted on *in vitro* preparations in order to understand the firing patterns of retinal neurons in response to both electrical and visual stimulations (Jensen et al., 2005; Sekirnjak et al., 2006). Although different authors have reported varying activation threshold values from *in vivo* studies (correlating to their respective electrode designs and animal models), together they provide strong evidence that electrical stimulation can elicit visual responses (Baig-Silva et al., 2005; Gekeler et al., 2004; Sachs et al., 2005). Numerous groups are now involved in developing and testing the feasibility of retinal prostheses (Chow and Chow, 1997; Eckmiller, 1997; Humayun et al., 1996; Mahadevappa et al., 2005). However, so far only a few studies have assessed the effects of prolonged stimulation on the morphology of retinal neurons (Colodetti et al., 2007; Guven et al., 2005; Nakauchi et al., 2007).

In our previous work, we observed a reduction in the thickness of the outer nuclear layer (ONL) caused by the pressure exerted by the electrode tip on the retina, with and without accompanying high charge stimulation (Colodetti et al., 2007). However, we found that the size of the disrupted region was statistically significantly larger when pressure was accompanied by high charge stimulation (Colodetti et al., 2007). We conducted the current study as part of our ongoing efforts to investigate the details of retinal cell injury in response to electrode pressure, with and without accompanying high charge stimulation. We found neural damage only in the outer retina, including a disturbance of synaptic vesicle proteins in the photoreceptor terminals and a remodeling of horizontal and rod bipolar cells' processes. In addition, we found no effect on inner retinal neurons. Similar results are present in areas adjacent to those directly contacted by the electrode tip.

2. Results

We began by first identifying the area directly contacted by the electrode as 2.5 mm away from the optic disc towards the nasal direction in each retina. The adjacent area was defined as the central retina area that was 1.5 mm away from the optic disc towards the nasal side. For experimental groups in which the electrode did not directly contact the retina, we used the same regions as described above in order to facilitate comparisons across groups. We observed no change in the thicknesses of the ONL, outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), and ganglion cell layer (GCL) between the normal and experimental groups in the central retina area adjacent to electrode contact (ONL, $38 \pm 1 \mu\text{m}$; OPL, $10 \pm 2 \mu\text{m}$; INL, $21 \pm 1 \mu\text{m}$; IPL, $48 \pm 1 \mu\text{m}$; GCL, $15 \pm 1 \mu\text{m}$ - Fig. 1A–E, M). We also did not observe any change in the thicknesses of retinal layers in the peripheral retinas of normal and experimental groups (ONL, $22 \pm 1 \mu\text{m}$; OPL, $5 \pm 1 \mu\text{m}$; INL, $15 \pm 1 \mu\text{m}$; IPL, $25 \pm 1 \mu\text{m}$; GCL, $10 \pm 1 \mu\text{m}$ -- Fig. 1F–J, N). However, consistent with our prior results, we did observe changes in the thicknesses of the ONL and OPL in Groups 3 (Fig. 1K, O) and 4 (Fig. 1L, P) in areas directly contacted by the electrode (ONL, $6 \pm 3 \mu\text{m}$ ($p < 0.0001$); OPL, $4 \pm 1 \mu\text{m}$ ($p < 0.0001$)). In these areas the thicknesses of INL ($21 \pm 1 \mu\text{m}$), IPL ($48 \pm 1 \mu\text{m}$) and GCL ($15 \pm 1 \mu\text{m}$) remained unchanged. Hence, we conclude that mechanical pressure alone, and mechanical pressure with high charge stimulation, both cause severe damage to the retinal area directly contacted by the electrode.

We next tested whether the synaptic properties of retinal neurons were affected by our experimental conditions in electrode-adjacent retinal areas, despite a lack of gross morphological change. We determined whether SV2A (Fig. 2A–G -- present in conventional synapses in the IPL and cone terminals (Wang et al., 2003)) and SV2B (Fig. 2H–N -- present only in the ribbon-synapse-containing terminals of bipolar cells, and of rods and cones (Wang et al., 2003)) showed normal expression patterns in experimental retinas. In normal (Fig. 2A, H), Group 1 (Fig. 2B, I), and Group 2 (Fig. 2C, J) retinas, SV2A and SV2B immunoreactivities were restricted to the OPL and IPL. In contrast, in Group 3 (Fig. 2D, K) and Group 4 (Fig. 2E, L) retinas, SV2A and SV2B-immunoreactive puncta were dispersed in the ONL (Fig. 2F, G, M, N). Although we observed abnormal expression of SV2A and SV2B in the outer retina, the expression pattern in the IPL was normal.

Since we found an abnormal expression of SV2A and SV2B in the outer retinas of Groups 3 and 4, we tested whether our experimental condition also altered processes of horizontal or rod bipolar cells in electrode-adjacent retinal areas (Fig. 3). Calbindin is a specific marker for horizontal cells and PKC α is a specific marker for rod bipolar cells in the mammalian retina (Haverkamp and Wässle, 2000). In normal (Fig. 3A), Group 1 (Fig. 3B), and Group 2 (Fig. 3C) retinas, the somata of calbindin-immunoreactive cells were strongly stained and found at the outer margin of the INL. Moreover, calbindin-immunoreactivity was also present in the OPL. The same results were apparent in Group 3 (Fig. 3D) and Group 4 (Fig. 3E) retinas, but in addition, calbindin antibodies revealed a sprouting of processes from horizontal cells, oriented towards the ONL (Fig. 3F, G). Also different from normal, in these groups the horizontal-cell somas were not round. We also found that the dendritic arbors of rod bipolar cells in the Group 3 (Fig. 3K) and 4 (Fig. 3L) retinas were abnormally oriented toward the middle of the ONL (Fig. 3M, N).

To determine whether the abnormal, radially extended processes of rod bipolar cells were coincident with the abnormal distribution of SV2B in the ONL, we performed double-labeling experiments with antisera against SV2B and PKC α (Fig. 4). In normal retinas (Fig. 4A), apical dendrites were confined to the OPL, where the photoreceptor terminals are present. In contrast, in Group 3 (Fig. 4B), the apical dendrites of rod bipolar cells were extended into the ONL, thus appearing in close apposition to the SV2B immunoreactivity. These results suggest that the apical dendrites of rod bipolar cells follow the abnormal distribution of SV2B into the middle of the ONL.

In the mammalian retina, most amacrine cells contain either glycine or γ -aminobutyric acid (GABA -- (Vardi and Auerbach, 1995)). Therefore, we performed labeling with antisera against the GABA synthesizing enzyme, glutamic-acid decarboxylase (GAD65), and glycine transporter-1 (Glyt-1 -- (Haverkamp and Wässle, 2000)) to probe for abnormalities in adjacent retinal areas (Fig. 5). We found no changes in the overall morphology and expression pattern of Glyt-1 (Fig. 5A–E) and GAD65 (Fig. 5F–J) immunoreactive amacrine cells. These results were consistent with the normal SV2A expression pattern that we found in the IPL for all groups (Fig. 2).

3. Discussion

In our previous study (Colodetti et al., 2007), we showed that mechanical pressure alone, and mechanical pressure with high charge stimulation, cause severe damage to the outer retina. In the present study, we note no obvious change in the gross morphology of areas adjacent to those directly contacted by the electrode, but do observe changes in SV2A and SV2B expression, as well as changes in horizontal cells and rod bipolar cells in these areas.

We further find similar synaptic vesicle expression patterns and retinal morphologies for the normal and experimental groups when there was no direct contact by the electrode on the retina. One reason for this could be due to under-estimation of the electrode-retina distance. Possible ways of testing this include different combinations of electrode-retina distance and stimulation charge intensities. Thus, along with intensity of stimulation, the distance of the electrode tip from the retina may be an important safety consideration.

Although the gross morphology of retinal areas adjacent to that contacted by the electrode was normal, we did observe abnormal distributions of SV2A and SV2B in the outer retinas of Groups 3 and 4 in the electrode-adjacent areas. This abnormal expression pattern of synaptic vesicle proteins in the outer retina may be due to retraction of photoreceptor terminals. The retraction of rod photoreceptor synapses is a feature that is evident in other overt insults of retinal tissues such as detached retinas (Fisher et al., 2005), and in diseases such as retinitis pigmentosa (Cuenca et al., 2005). The structural changes evident in these studies are associated with overt damages in photoreceptor cells. Double labeling showed very close contact between the dendritic trees of rod bipolar cells and photoreceptor terminals in the middle of the ONL. Similar changes in rod bipolar cells dendrites have been noted in age-related macular degeneration (Sullivan et al., 2007), in detached retinas (Fisher et al., 2005), and in retinitis pigmentosa (Cuenca et al., 2005) due to retraction of photoreceptors terminals.

On the other hand, GAD65- and Glyt-1-immunoreactive amacrine cells did not appear to be affected by our experimental conditions in electrode-adjacent areas (Fig. 5). We do not yet have any evidence that amacrine cells are affected in the areas directly contacted by the electrode in Group 3 and 4 retinas. In addition, we did not see any change in GCL thickness (Fig. 1). Despite electrode placement on the ganglion cell side, and the high charge stimulation, ganglion cells seemed unaffected. This suggests that an epiretinal electrode array placed on a degenerate retina may be tolerated well. It is somewhat surprising that although the mechanical pressure occurred close to the GCL, the inner retinas were not severely affected, but morphological changes were observed in the outer retina (Fig. 1K, L). In a number of retinal disorders, including macular degeneration, retinal detachment (RD), diabetic retinopathy, retinopathy of prematurity, and retinitis pigmentosa (RP), photoreceptors are highly vulnerable and undergo apoptosis (Cook et al., 1995; Arroyo et al., 2005). Recently, it has been noted that Monocyte chemoattractant protein 1 (MCP1) plays a critical role in mediating photoreceptor apoptosis in an experimental model of RD. RD led to increased MCP1 expression in the Muller glia and increased CD11b macrophage/microglia in the detached retina (Nakazawa et al., 2006). In our previous study (Colodetti et al., 2007), we showed increased GFAP expression in Group 3 and Group 4. Thus, it is conceivable that MCP1 levels may have increased in the glia to cause cell death in photoreceptor cells in Group 3 and Group 4 in areas directly contacted by the electrode.

Clinical trials in epiretinal stimulation have shown the feasibility of eliciting phosphenes, providing some improvement in vision (Humayun et al., 1996; Mahadevappa et al., 2005). Numerous groups worldwide at present are involved in developing sophisticated implants that will stimulate the retina at many different locations. The ultimate goal of all such implants is to provide patients with visual percepts that closely resemble normal vision. This will in turn require implants to function for prolonged periods of time. Additionally, high intensity electrical stimulation in other neuronal structures has been shown to cause irreversible tissue damage in some cases, or a temporary desensitization in others, based upon the electrode design, stimulus parameters and the properties of the target neurons (McCreery et al., 1990; McCreery et al., 1995; McCreery et al., 2002; McCreery, 2004). Thus, a study towards understanding the consequences of long-term stimulation of the retina via such implants is warranted. The current study is an attempt to explore the safety limits associated with the mechanical pressure exerted by an electrode and high intensity electrical stimulation. In

summary, we show morphological changes in retinal cells of the rat retina in response to pressure exerted by the electrode, with and without accompanying high charge stimulation. These results suggest that retinal implants themselves can cause damage to the outer retina by exerting physical pressure that is not well tolerated by the retina.

4. Experimental Procedure

4.1 Animals

Twenty adult Long Evans pigmented rats (Charles River Laboratories, Inc., 251 Ballardvale Street, Wilmington, MA 01887) were used ($\approx$ 5 month old) with experiments performed on one eye in each animal. All the tissue used here was collected as part of the previous study (Colodetti et al., 2007). For each experimental condition, four to five rats were used: normal, Group 1, Group 2, Group 3, and Group 4 (please see Experimental Groups). All animals were maintained on a daily 12 h light/dark cycle. All procedures were in conformance with the Guide for Care and Use of Laboratory Animals (National Institutes of Health). The University of Southern California Institutional Animal Care and Use Committee reviewed and approved all procedures.

4.2 Experimental groups

There are four experimental groups: Group 1 (stimulating electrode near, but not contacting the retina – 0.00 μC of charge per phase); Group 2 (stimulating electrode near, but not contacting the retina – 0.09 μC of charge per phase); Group 3 (stimulating electrode directly contacting the retina – 0.00 μC of charge per phase); Group 4 (stimulating electrode directly contacting the retina – 0.09 μC of charge per phase); and one normal group representing normal retina. The level of stimulus charge chosen is in the range of stimulus levels reported for human clinical trials (Mahadevappa et al., 2005). The timing of the pulses was the same for all experiments: 1 ms cathodic, 1 ms delay, 1 ms anodic, applied at 100 pulses/s. The corresponding charge density was estimated to be 0.09 $\mu\text{C} = 1 \text{ mC}/\text{cm}^2$, based on the geometry of the stimulating electrode (description of electrode is given below). Charge/phase (not charge density) is used to describe the stimulus in this paper, since the electrodes were hand-made and had some variability in surface area ($\pm 5\%$, based on estimates from manufacturer, see next paragraph). This charge density was above the safe limit for chronic stimulation for platinum (Brummer et al., 1983), since the intent of the study was to investigate the effect of high charge density stimulation.

4.3 Electrical Stimulation Setup

For a detailed explanation of the stimulating electrode and experimental setup, please refer to our previous work (Colodetti et al., 2007). In brief, the stimulating electrode was the inner pole (Pt/Ir) of a concentric bipolar electrode (model CBDRG75, FHC Inc. Bowdoin, ME, USA). The diameter of the inner pole was 75 μm with a round tip configuration. A needle electrode inserted subcutaneously in the back of the animal was used as the return path for the stimulus current. Cathodic-first, charge-balanced biphasic pulses were generated using a stimulus generator (STG 2008, Multi Channel Systems, Reutlingen, Germany) fed to an analog voltage-to-current converter (Model 2200, A-M System, Carlsborg, USA). Care was taken to ensure that there was no DC offset during stimulation. Prior to the start of the experiment, electrochemical measurements (impedance and cyclic voltammetry) were performed to check the integrity of the electrode.

4.4 Surgical Procedure

Animals were anaesthetized through intramuscular injection of 4 parts ketamine hydrochloride 10% 50–90 mg/kg (Ketaset, Fort Dodge Animal Health, Fort Dodge, IA, USA) and 1 part xylazine 2% 5–10 mg/kg (X-ject E, Phoenix Scientific Inc., St. Joseph, MO, USA).

The eye was dilated using 1% tropicamide and 2% phenylephrine. The anaesthetized rat was positioned on a heater pad set at 37°C to maintain body temperature. The eye was proptosed through a 4 × 4 mm hole of a 2 × 2 cm section of a sterile latex surgical glove to improve surgical exposure of the eye. The retina was viewed through the microscope by slightly flattening the cornea with an 18 × 18 mm glass coverslip. The stimulating electrode was inserted through a temporal sclerotomy that was made using a 27 gauge needle electrode and positioned towards the nasal side. The stimulating electrode was mounted onto a 1 cc syringe prior to insertion and held in position by a single-axis micropositioner stage. For the experimental groups where the electrode tip contacted the retina (Groups 3 & 4), the electrode tip was advanced slowly towards the retina and the position was fixed when a slight dimpling of the retina could be observed (≈2.5 mm away from the optic disc towards the nasal direction). For the experimental groups where no contact was made with the retina (Groups 1 & 2), the electrode tip was positioned close to the retina, and the shadow cast on the fundus by the electrode was used to estimate the proximity to the retina. The electrode position was verified every 15 minutes through indirect ophthalmoscopy. After completion of the experiment, the electrode was removed and the health of the animal was monitored throughout the post surgical recovery period. Different diagnostic tests were carried out during the recovery period. For a more detailed explanation of the surgical procedure and diagnostic tests, please refer to our previous work (Colodetti et al., 2007).

4.5 Tissue Preparation

The animals were euthanized 2 weeks post-operatively and their eyes were enucleated. During enucleation, a cut was made on the superior retina in order to preserve orientation information during histological analysis. The anterior segments of the enucleated eyes were then removed and the eyecups were fixed by immersion in Bouin's fixative (picric acid, formalin and acetic acid; Polysciences, Inc, PA) on a shaker for 2 – 5 h at room temperature. Following fixation, eyecups were then washed by several changes of PB and transferred to 80% ethanol at 4 °C. The eyecups were then embedded in paraffin, continuing from 80% ethanol. Eyecups were sectioned in serial order along the vertical meridian on a microtome at a thickness of 5 µm. Sections were collected on gelatin-coated slides for immunocytochemistry and hematoxylin staining. The paraffin from the sections was removed by xylene.

4.6 Hematoxylin staining

For hematoxylin staining, slides were dipped in hematoxylin for 5 min. They were then washed in tap water, dehydrated in alcohol, cleared in xylene, and mounted in xylene-based medium (Richard-Allan Scientific, Kalamazoo, MI, USA). Every fifth slide was chosen for hematoxylin staining.

4.7 Immunocytochemistry

For fluorescence immunocytochemistry, 5-µm-thick retinal sections were incubated in 10% normal donkey serum (NDS) and 1% Triton X-100 in PBS for 1 h at room temperature. Sections were then incubated overnight with a rabbit polyclonal antibody directed against Calbindin (Swant, Bellinzona, Switzerland, dilution 1:1000); PKCα (Sigma, dilution 1:1000); GAD65 (Chemicon, dilution 1:1000); SV2A and SV2B (Synaptic systems, Goettingen, dilution 1:2000); a mouse monoclonal antibody directed against PKCα (BD Biosciences Pharmingen, dilution 1:1000); a goat polyclonal antibody directed against glycine transporter 1 (Glyt-1)

(Chemicon, dilution 1:8000). Each antiserum was diluted with PBS containing 0.5% Triton X-100 at 4 °C. Retinas were washed in PBS for 45 min (3×15 min) and afterwards, incubated for 2 h in carboxymethylindocyanine (Cy3)-conjugated affinity-purified, donkey anti-rabbit IgG (Jackson Immuno Labs, West Grove, PA, dilution 1:500); or Alexa 488, anti-mouse IgG or anti-goat (1:300 dilution; Molecular Probes, Eugene, OR) at room temperature. Retinal sections were washed for 30 min with 0.1 M PB and coverslipped with Vectashield mounting media (Vector, Burlingame, CA). Sections were then analyzed using a Zeiss LSM 510, (Zeiss, NY) confocal microscope. Alexa 488 labeling was excited using the 488-nm line of an Argon ion laser. For the detection of the Cy3 signal, the 543-nm line of a green HeNe1 laser was used. Immunofluorescence images were processed in Zeiss LSM-PC software. The brightness and contrast of the images were adjusted using Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA). For presentation, all Photoshop manipulations (brightness and contrast only) were carried out equally across sections.

For double labeling, same procedure was followed as above except for incubating two primary (PKC α and SV2B) and secondary (Alexa 488-mouse and Cy3-rabbit) antibodies together.

4.8 Statistical analysis

Student's t-tests were used for comparisons within Group 3 and Group 4, and between Groups. The t-tests were used to examine the difference between two means in the thickness measurements of retinal layers ($n=5$, two-sided test), and extent of damaged areas ($n=5$, one-sided test). All statistical tests were performed using Stat View (Abacus Concepts, Berkeley, CA, USA). A difference between the means of separate experimental conditions was considered statistically significant at $P < 0.05$.

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Figure 1.

Photographs taken from 5- μm -thick vertical sections processed by hematoxylin staining. Hematoxylin staining in normal (A, F) and experimental groups (Group 1 - B, G; Group 2 - C, H; Group 3 - D, I; Group 4 - E, J) showed no detectable changes in the thicknesses of electrode-adjacent area of retina (A-E, M -- 1.5 mm away from the optic disc towards the nasal side). This held for the peripheral (F-J, N) of the retinas. However, lesion areas were noted in the directly electrode-contacted area of retina (K, L-- $\approx$ 2.5 mm away from the optic disc towards the nasal side). The thickness of the ONL and OPL in Group 3 and 4 was significantly reduced in retinal areas directly contacted by the electrode (O, P--the symbol * represents $p < 0.0001$). ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Data represents mean $\pm$ standard deviation. Scale bars = 50 μm .

Figure 2.

Photographs of vertical sections of electrode-adjacent retinal areas processed for SV2A (A–G) and SV2B (H–N) immunoreactivity in normal (A, H) and experimental groups (Group 1 – B, I; Group 2 – C, J; Group 3 – D, K; Group 4 – E, L). A–C: Labeling for SV2A was present in synaptic terminals in the OPL and IPL. D, E: Labeling for SV2A was dispersed in ONL. H–L: Labeling for SV2B was present in the OPL and IPL. K, L: Labeling for SV2B was dispersed in ONL. Scale bar = 50 μ m. F, G: Higher-power microphotographs of the same retinal field as in D and E. M, N: Higher-power microphotographs of the same retinal field as in K and L. Scale bar = 20 μ m.

Figure 3.

Photographs of vertical sections of electrode-adjacent retinal areas processed for calbindin (A–G) and PKC α (H–N) immunoreactivity in normal (A, H) and experimental groups (Group 1 – B, I; Group 2 – C, J; Group 3 – D, K; Group 4 – E, L). A–C: Labeling for calbindin was present in horizontal cells and OPL. D, E: Labeling for calbindin showed sprouting processes oriented toward the ONL. H–L: Labeling for PKC α was present in the rod bipolar cells. K, L: Dendritic trees of rod bipolar cells was present in middle of the ONL. Scale bar = 50 μ m. F, G: Higher-power microphotographs of the same retinal field as in D and E. M, N: Higher-power microphotographs of the same retinal field as K and L. Scale bar = 20 μ m.

≈1.5 mm away from the optic disc towards the nasal side

Normal

Group 3

Figure 4.

Confocal micrographs of a vertical retinal section of electrode-adjacent retinal areas processed for SV2B (green) and PKCα (red) immunoreactivity in normal (A) and Group 3 (B) retinas.

A: Double exposure shows close contact between SV2B and PKCα at the OPL (*arrows*). **B:** Double exposure shows close contact between SV2B and PKCα at the OPL (*arrow*) and middle of the ONL (*arrowheads*). Scale bar = 10 μm.

Figure 5.

Photographs of vertical sections of electrode-adjacent retinal areas processed for Glyt-1 (A–E) and GAD65 (F–J) immunoreactivity in normal (A, F) and experimental groups (Group 1 – B, G; Group 2 – C, H; Group 3 – D, I; Group 4 – E, J). A–E: Labeling for Glyt-1 was present in numerous amacrine cells in the INL and processes in the IPL. F–J: Labeling for GAD65 was present in numerous amacrine cells in the INL and processes in the IPL. Expression pattern of Glyt-1 and GAD65 was consistent in normal and experimental conditions. Scale bar = 50 μ m.