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Dopaminergic amacrine cells express opioid receptors in the mouse retina

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Abstract

The presence of opioid receptors has been confirmed by a variety of techniques in vertebrate retinas including those of mammals; however, in most reports the location of these receptors has been limited to retinal regions rather than specific cell-types. Concurrently, our knowledge of the physiological functions of opioid signaling in the retina is based on only a handful of studies. To date, the best documented opioid effect is the modulation of retinal dopamine release, which has been shown in a variety of vertebrate species. Nonetheless, it is not known if opioids can affect dopaminergic amacrine cells (DACs) directly, via opioid receptors expressed by DACs. This study, using immunohistochemical methods, sought to determine whether (1) μ - and δ -opioid receptors (MORs and DORs, respectively) are present in the mouse retina, and if present, (2) are they expressed by DACs. We found that MOR and DOR immunolabeling was associated with multiple cell-types in the inner retina, suggesting that opioids might influence visual information processing at multiple sites within the mammalian retinal circuitry. Specifically, colabeling studies with the DAC molecular marker anti-tyrosine hydroxylase antibody showed that both MOR and DOR immunolabeling localize to DACs. These findings predict that opioids can affect DACs in the mouse retina directly, via MOR and DOR signaling, and might modulate dopamine release as reported in other mammalian and non-mammalian retinas.

Keywords

μ -opioid receptor; δ -opioid receptor; dopamine; amacrine cell; mouse

Introduction

Endogenous opioids play an important role in processing sensory information such as pain (Akil et al., 1984; Pan et al., 2008), but only sporadic data suggest that endogenous opioids are present in the mammalian retina: enkephalin was detected in inner retinal neurons of guinea pigs (Altschuler et al., 1982) and in rat retinal extract (Peng et al., 2009), and we recently demonstrated the expression of β -endorphin in cholinergic amacrine cells in mouse (Gallagher et al., 2010). The three classes of opioid receptors do not show exclusive endogenous substrate specificity, however, β -endorphin binds preferentially to μ -opioid receptors (MORs), enkephalins to δ -opioid receptors (DORs) and dynorphins to κ -opioid receptors (KORs) (Kieffer, 1995). Out of these three receptor classes, binding studies with [3 H]dihydromorphine indicated autoradiographic labeling in the inner plexiform and ganglion cell layers (IPL and GCL, respectively), suggesting the presence of MORs and/or DORs in rat and monkey retinas (Wamsley et al., 1981). In rat retina, Peng et al. (2009) showed the presence of both MORs and DORs through RT-PCR and Western blot analysis,

and MORs were also detected by immunohistochemistry on processes of bistratified ganglion cells (Brecha et al., 1995).

In the mammalian retina opioids regulate cell proliferation during development (Isayama & Zagon, 1991), influence cell survival following hypoxic or ischemic challenge (Husain et al., 2009; Peng et al., 2009; Riazi-Esfahani et al., 2009) and regulate dopamine release via DOR and MOR activation (Dubocovich & Weiner, 1983). As dopamine—released from dopaminergic amacrine cells (DACs)—exerts action in a paracrine fashion on most retinal cell-types to promote adaptation to bright light conditions (Witkovsky, 2004), opioid regulation of dopamine release could have profound physiological consequences in the retinal circuitry.

The aim of this study was to investigate the presence and the location of opioid receptors in the mouse retina with immunohistochemical methods. Here we show that MOR and DOR immunolabeling is associated with ganglion- and GABAergic amacrine cells, including DACs. We propose that in the mouse retina β -endorphin, released from cholinergic amacrine cells (Gallagher et al., 2010), acts on MORs (and perhaps DORs) relatively close to its release site in the inner retina, and might affect visual processing by amacrine, and ganglion cells, much like substance P (Brecha et al., 1989; Zalutsky & Miller, 1990). Specifically, the results of this study predict that in the mouse retina endogenous opioids can exert their effect via direct action on MORs and DORs expressed by DACs and might modulate dopamine release.

Materials and methods

Animals

Adult male and female wild-type C57 and C57BL/6J mice, GAD67-EGFP transgenic mice (Tamamaki et al., 2003) and Sprague-Dawley dams were used for experimentation. Animals were handled in compliance with the Colorado State University Institutional Animal Care and Use Committee and all procedures met United States Public Health Service Guidelines. All efforts were made to minimize the number of animals used and any possible discomfort. Mice were obtained from Jackson Laboratories, Bar Harbor, ME, and rats from Harlan Laboratories, Indianapolis, IN. Animals were kept on a 12 hr light:12 hr dark cycle with lights on at 6:00 AM, fed standard chow and water *ad libitum*.

Immunohistochemistry

Immunohistochemical procedures on retina-, brain-, and dorsal root ganglia (DRG)-sections were conducted as previously described for retinal sections (Gallagher et al., 2010), except an antigen retrieval step (15 min in boiling 10 mM sodium citrate) followed by 0.5% sodium borohydride treatment for 45 min was included. Brain slices were prepared from anesthetized (i.p. 0.1 – 0.15 ml of 50 mg/ml Beuthanasia-D (Schering-Plough Animal Health)) mice transcardially perfused with 0.1 M phosphate buffer (PB) and 4% paraformaldehyde (PFA) in PB. After perfusion brain was removed, post-fixed for 1-2 hrs, cryoprotected and sectioned (50 μ m). Rats were anesthetized with isoflurane and euthanized via decapitation. DRGs were removed, fixed in 4% PFA, cryoprotected and sectioned (20 μ m).

Antibodies

Antibody raised against Brn-3a—This goat anti-Brn-3a antibody (C-20) was generated against a synthetic peptide corresponding to the N-terminus region of human Brn-3a (Santa Cruz Biotechnology: sc-31984). Western blot analysis of rat retina lysate yielded a 48 KD

band (Nadal-Nicolás et al., 2009). In the mouse retina, this antibody has been used to exclusively label retinal ganglion cells (Galindo-Romero et al., 2011).

Antibodies raised against δ -opioid receptors (DORs)—The first rabbit anti-DOR antibody was generated against a synthetic peptide corresponding to amino acids 2-18 (ELVPSARAEQLQSSPLVN) of the N-terminus of mouse DOR (Alomone Labs: AOR-014 / AN-01). Western blot analysis of rat cortex lysate showed bands representing receptor monomers (37-43 kD) and dimers / oligomers (>75 kD) which were absent in experiments preincubated with control peptide (manufacturer's specifications). This antibody was used in mouse DRG, showing immunolabeling of both large and small neurons which was abolished via preadsorption with control peptide and absent in DOR knock-out mice (Wang et al., 2010).

The second polyclonal rabbit anti-DOR antibody (Millipore: AB1560 / LV1480422) used in this study was raised against the N-terminus of mouse DOR (LVPSARAEQLQSSPLV). Western blot analysis of adult rat brain homogenate identified bands representing receptor monomers, dimers, and possible oligomers, which were blocked with preadsorption in control peptide (Persson et al., 2005). In rat, this antibody has been shown to label DOR+ and large, medium, and small neurons in DRG (Kabli & Cahill, 2007). In our hands, this anti-DOR antibody showed similar and appropriate labeling in cryostat sectioned rat DRG (data not shown).

Antibodies raised against μ -opioid receptors (MORs)—The first rabbit anti-MOR antibody used in this study was generated against a peptide corresponding to amino acids 22-38 (CSPAPGSQLNLNLSHVDGN) of the extracellular N-terminus of rat MOR (100% homology with that of mouse) (Alomone Labs: AOR-011 / AN-01). Western blot analysis of rat hippocampus lysate showed a band of 55-60 kD (manufacturer's specifications).

The second rabbit anti-MOR antibody (Epitomics: 3675-1 / H101201) was raised against a synthetic peptide corresponding to amino acids 386-398 (LENLEAETAPLP) of the intracellular C-terminus of mouse MOR. Western blot analysis of mouse brain homogenates resulted in a broad band labeling of ~70-80 kDa in wild-type but not in knockout mouse preparations (Lupp et al., 2011). In our hands, this antibody showed similar immunolabeling in mouse hippocampus (data not shown) as seen in rat (Lupp et al., 2011).

Antibody raised against Tyrosine Hydroxylase (TH)—The mouse anti-TH monoclonal antibody (Millipore: MAB318 / LV1556893) was generated against TH purified from PC12 cells, and its characterization in mouse retina has been previously described (Gallagher et al., 2010).

Data from images

Confocal laser microscopy—Fluorescent images were taken with a Zeiss LSM 510 confocal microscope (Carl Zeiss, Oberkochen, Germany). For all acquisitions, sequential scans at the different wavelengths were performed. Z-stack images through the full thickness of immunolabeled tissues were taken at 40x, 2-5 μ m increments. Brightness and contrast of images were adjusted uniformly in Photoshop CS3 (Adobe 10.1). Images were compiled and analyzed using Zeiss LSM Image Examiner software (Carl Zeiss, Oberkochen, Germany).

Quantification and data analysis—Compiled single-plane (“Z-stack”) images were used for subjective, visual assessment of immunolabeling colocalization. Quantitative analysis of opioid receptor colocalization with TH immunolabeling was performed on single-plane confocal images through the center of TH+ cell bodies or processes (see dashed

lines in Fig. 2C) using Image J software (NIH, Bethesda, MD, USA). The JACoP plug-in was used to calculate the Pearson's coefficient $\pm$ SEM (Bolte & Cardelières, 2006) using the Costes' approach. Pearson's coefficient (PC) provides an analysis of pixel intensity and location in a dual-channel image with values ranging from -1 to 1 (-1: negative correlation; 0: no correlation; 1: complete correlation) (Gonzalez & Wintz, 1987). The Costes' approach sets an automatic threshold level for both channels to eliminate inconsistent or irreproducible results. Furthermore, it provides a statistical probability for disrupting the level colocalization (PC) found on a given two channel image by randomizing the pixels independently 1000 times. In practice, $P > 95\%$ indicates that a colocalization pattern is non-random (Costes et al., 2004; Bolte & Cardelières, 2006).

Results

μ -opioid receptors in the mouse retina

To assess the immunohistochemical labeling of μ -opioid receptors (MORs) in the mouse retina, an antibody recognizing the N-terminus of MOR (Alomone) was tested on vertical cryostat sections. This antibody at 1:500 dilution produced punctate labeling in the inner retina which was associated with somatic profiles in both the inner nuclear layer (INL) and ganglion cell layer (GCL), but no immunolabeling was noted in the outer retina (arrows, Fig. 1A). MOR immunolabeling was completely abolished by preincubation of the antibody with its control peptide (1:10, antibody: control peptide—per manufacture's guidelines) (Fig. 1B). Control experiments were performed on mouse brain coronal sections. MOR immunolabeling of hippocampal neurons corresponded nicely with previous reports in mouse (Kwon et al., 2008) (Fig. 1C,D). Similar to the retina, preadsorption of the MOR antibody with its immunogenic peptide blocked the immunolabeling of hippocampal neurons (Fig. 1E).

The GCL is comprised of ganglion cells (GCs) and displaced amacrine cells (Jeon et al., 1998; Kong et al., 2005), whereas the INL is the most heterogeneous nuclear layer in the mouse retina containing horizontal, bipolar, amacrine and Müller cell somas, with amacrine making up a large fraction (~39%) of all the INL somata (Jeon et al., 1998). Due to the diverse size, morphology and location of the MOR+ somas (Fig. 1A), it was likely that there are multiple types of MOR bearing cells in the inner retina. In a GAD67-EGFP knock-in mouse line, the presence of EGFP reliably marks GAD67 positive GABAergic neurons in the central nervous system (Tamamaki et al., 2003) including various GABAergic amacrine cells the retina (May et al., 2008). We found that MOR immunolabeling occasionally colocalized with the GAD67-EGFP signal in both the INL and GCL (Fig. 2A, asterisk and arrow, respectively). The presence of MOR+/GAD67-EGFP- cells in the GCL (Fig. 2A, arrowheads) indicated that displaced amacrine lacking GAD67-EGFP and/or GCs express MORs as well. To further test this notion, colabeling studies were performed using a known GC marker, Brn-3a (1:200 dilution), which label many but not all GCs in the mouse retina (Xiang et al., 1995). We found several Brn-3a labeled cell bodies in the GCL colocalize with MOR immunolabeling (Fig. 2B, arrow), but not all Brn-3a cells were colabeled with MOR (Fig. 2B, arrowhead).

To evaluate whether DACs are also MOR+, a colabeling study was performed with the DAC marker anti-tyrosine hydroxylase (TH) antibody (Witkovsky et al., 2005). A representative image (Fig. 3A-C) shows colocalization of MOR and TH immunolabeling in the INL (arrow). Visual evaluation of 124 TH+ DAC somas in retinal sections from nine different mice showed that 117 (> 94%) were also MOR+.

A more objective test to assess whether MOR and TH immunolabeling precisely overlap is to calculate Pearson's coefficient (PC) using Costes' approach (see Methods). Analysis of

five MOR / TH immunolabeled somata (see Fig. 3C dashed line for example of focused somatic colocalization analysis) from three animals gave an average PC of 0.603 ± 0.015 ; $P = 100\%$. This PC indicates colocalization between TH and MOR and the P-value proves that it is non-random. Similar analysis of three TH+ DAC somata that were deemed as MOR- by visual assessment was also performed. The results of this analysis ($PC=0.4 \pm 0.094$; $P = 100\%$) suggest that our (subjective) visual assessment was too conservative and underestimated the colocalization percentage; based on these results in the mouse retina essentially every (TH+) DAC soma was labeled with the MOR antibody directed against the N-terminus.

Processes from DACs form a distinct horizontal band at the border of the INL and IPL (Witkovsky et al., 2005). Both visual assessment and quantitative analysis of colocalization were performed on five images focused on TH+ processes (see Fig. 3C IPL dashed line for example) yielding little to no colocalization with MOR immunolabeling ($PC=0.062 \pm 0.006$).

Studies with a second anti-MOR antibody directed against the C-terminus of MOR (1:10 dilution) showed similar immunolabeling in the inner retina as was seen with the N-terminus-directed MOR antibody (compare Fig. 3D with 1A and 3A). Additionally, this second MOR antibody provided consensus colabeling with TH (arrow, Fig. 3D-F). We found $> 93\%$ of TH+ somas were MOR+ by visual assessment (28/30, four mice), whereas no colocalization was noted in the IPL. Detailed statistical evaluation of colocalization was performed on five images focused on TH+ cell bodies from three animals resulted in a PC of 0.754 ± 0.025 , with a P-value of 100%, indicating a non-random colocalization between TH and MOR immunolabeling provided by the C-terminus antibody.

δ -opioid receptors in the mouse retina

The physiological data in rabbit retina showed that DADLE reduces dopamine release (Dubocovich & Weiner, 1983). DADLE, a synthetic enkephalin, is considered to be a δ -opioid receptor (DOR)-selective agonist (Kosterlitz et al., 1980). Therefore, cryostat sectioned mouse retinal tissue was labeled with an antibody directed against the N-terminus of DOR (Alomone). This antibody (1:500 dilution) provided punctate immunolabeling in the inner retina, with the strongest signal seen in the INL (arrows, Fig. 4A). Preadsorption of the antibody with its control peptide (1:10, antibody: control peptide—per manufacture's guidelines) completely blocked labeling (Fig. 4B). Immunohistochemical studies of DOR distribution have been performed extensively in the rat dorsal root ganglia (DRG). In our hands this anti-DOR antibody also labeled neurons within the rat DRG (Fig. 4C) consistent with published data (Kabli & Cahill, 2007), which was completely abolished by preadsorption with its control peptide (Fig. 4D). Taken together, the DOR immunolabeling provided by the anti-DOR antibody from Alomone appeared to be specific both in the DRG and in the retina.

Colabeling studies with anti-TH antibody indicated that one of the DOR+ retinal cell types is the DAC (Fig. 5A-C, arrow). Out of 36 TH+ DAC somata analyzed from retinal sections of three mice, 35 ($> 97\%$) were judged to be DOR+ by visual assessment. Detailed colocalization analysis of five TH / DOR labeled cells from three animals resulted in a PC of 0.699 ± 0.052 and a P-value of 100%. These values indicate a strong and non-random somatic colocalization of DOR and TH immunolabeling in mouse retina.

Analysis of DOR and TH immunolabeling colocalization was also performed for TH+ processes. By visual assessment no colocalization was detected within the IPL. Computer based analysis of five images focused on TH+ processes in the IPL from three mice gave a PC of -0.058 ± 0.018 . Although negative, the near-zero PC implied non-colocalization of

TH and DOR immunolabeling in the mouse IPL—suggesting that DOR labeling was limited to DAC somas.

Similar results were obtained by using a second anti-DOR antibody from Millipore. This DOR antibody (1:500-1:600 dilution) labeled robustly within the inner retina, including somatic labeling within the INL (Fig. 5D, arrowhead) and Brn-3a+ GC somas in the GCL (Fig. 5D, arrow). Colabeling studies with DOR and TH antibodies in retinal sections made from GAD67-EGFP mice showed corresponding colocalization of DOR and TH (Fig. 5E-F). Note, that the GAD67-EGFP signal does not colocalize with TH, consistent with the notion that DACs might use GAD65 to generate GABA (May et al., 2008; Witkovsky et al., 2008). Out of 62 TH+ cell bodies from six mice > 93% (58/62) were also deemed DOR+ by visual assessment. Detailed statistical evaluation of colocalization was performed on five images focused on TH+ cell bodies from five animals and resulted in a PC of 0.767 ± 0.032 and a P-value of 100%, confirming that the labeling pattern produced by second anti-DOR antibody also colocalized with that of anti-TH antibody, in a non-random manner. Although not investigated further in the current study, it is important to note the presence of DOR+, GAD67-EGFP+ displaced ACs (Fig. 5E, arrow) in the mouse retina.

Discussion

In the current study we demonstrate MOR immunolabeling in the mouse retina, for the first time showing strong somatic labeling in the INL and GCL (Fig. 1A). Although systematic classification of all MOR+ somas was not attempted in this study, our data indicates that in the mouse retina a subpopulation of Brn-3a+ GCs (Fig. 2B), GAD67+ GABAergic ACs (Fig. 2A), and dopaminergic amacrine cells (DACs) express MORs (Fig. 3). Similarly, DOR immunolabeling in the mouse retina implicated multiple DOR+ inner retinal cell-types (Fig. 4A), including Brn-3a+ GCs (Fig. 5D) and GAD67-expressing GABAergic amacrine cells (Fig. 5E). Importantly, our data confirms that DACs are DOR+ (Fig. 5A-C, E-F).

TH+ dopaminergic processes showed neither MOR nor DOR immunolabeling. This labeling pattern is not unprecedented: natriuretic peptide receptor labeling was also associated primarily with the somatic region of DACs (Abdelalim & Tooyama, 2010). Nonetheless, it is not known whether the somatic punctate labeling is associated with functional neuropeptide receptors in the plasma membrane or with newly synthesized receptor protein in the cytoplasm.

The majority of opioid receptor activity is mediated through the Go/Gi -coupled superfamily of receptors, and the cellular effects include: (1) activation of inwardly rectifying potassium current; (2) inhibition of voltage-gated calcium current; and (3) inhibition of adenylate cyclase, depending on the actual cell-type (Kieffer, 1995). Consequently, opioid receptor activation is generally believed to be inhibitory at cellular level—often expressed as a reduction of transmitter release from neurons possessing opioid receptors, such as reduction of dopamine release in the striatum (Loh et al., 1976).

Morgan & Boelen (1996) proposed an intercellular feedback loop in the avian retina formed by the endogenous opioid system and DACs that mediates dark-light switch. Considering that besides birds (Su & Watt, 1987), retinal dopamine release is reduced by opioids in multiple species (turtle: Kolbinger & Weiler, 1993; rabbit: Dubocovich & Weiner, 1983), this model might be more generally applicable to vertebrate retinas. Here, MOR and DOR immunolabeling was found to be associated with (TH+) DACs in the mouse retina, which predicts that opioids modulate the function of DACs directly, via MORs and DORs located on DACs and might influence dopamine release as in other species.

Whether these opioid receptors are coexpressed in any other retinal cell-type besides DACs requires further study. However, in other parts of the mammalian nervous system, in cell-types that coexpress MOR and DOR, MOR/DOR heteromerization have been shown to modulate signaling (Gomes et al., 2004; Rozenfeld & Devi, 2011). Besides DACs, we found that MOR and DOR immunolabeling was associated with a heterogeneous cell population in the inner retina including GABAergic displaced amacrine and ganglion cells. These findings suggest that opioids might affect retinal function at multiple sites of action, thus our work adds to a framework on which future histological and physiological characterizations can occur.

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Figure 1.

Immunohistochemical localization of MORs in mouse retinal and hippocampal tissues. **A:** 40x confocal single-plane image of vertical cryosectioned mouse retina showing immunolabeling with anti-MOR antibody directed against the N-terminus of MOR (Alomone). MOR+ puncta are observed in the inner retina with discernible cells labeled in the INL and GCL (arrows). **B:** 40x image similar to **A** showing control peptide preadsorption for MOR antibody. **C:** 10x confocal image of mouse brain slice focusing on the hippocampus. MOR antibody showing appropriate immunolabeling (green), colabeled with the nuclear marker ToPro3 (red). **D:** 40x focused confocal image of the CA3 region of mouse hippocampus immunolabeled for MORs (green), colabeled with ToPro3 (red). **E:** 40x image similar **D** showing preadsorption of MOR antibody with control peptide, colabeled with ToPro3 (red). ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer; DG: dentate gyrus. Scale bars: **A, B, D, and E:** 20 μ m, **C:** 100 μ m.

Figure 2.

Some ganglion cells and GABAergic amacrine cells are MOR+. **A:** 40x single-plane merged image of vertically sectioned GAD67-EGFP mouse retina immunolabeled for MOR (red; Alomone). GAD67-EGFP somas are seen in the INL (bright green) and GCL (dim green) with processes in the IPL. Punctate MOR+ labeling of a displaced GABAergic amacrine cell is shown in the GCL (arrow). Some putative MOR+ somas in the GCL are GAD67-EGFP negative (arrowheads). In the INL some GAD67-EGFP cells colabel MOR+ puncta that could indicate colocalization (asterisks). **B:** 40x merged confocal image of cryosectioned wild-type mouse retina co-immunolabeled for MOR (red) and Brn-3a (green). Some Brn-3a + retinal ganglion cells (arrow), but not all (arrowhead), are MOR+. INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. Scale bars: 20µm.

Figure 3.

Dopaminergic amacrine cells in the INL are MOR+. **A:** 40x single-plane image of vertically sectioned mouse retina showing MOR+ (red) puncta in the INL (arrow) using the N-terminus directed MOR antibody (Alomone). **B:** Image displaying the same retinal region as **A**, immunolabeled for TH (green) showing a single TH+ cell (arrow) in the INL with TH+ projections in the IPL at the border with the INL. **C:** A merged image of **A** and **B**, displaying colocalization of the MOR+ and TH+ cell (arrow). **D:** 40x confocal image, vertical section of mouse retina showing immunolabeled somata (red) in the INL (arrow) with the anti-MOR antibody directed against the C-terminus of the receptor (Epitomics). **E:** Image illustrating the same region as in **D**, showing a TH+ (green) soma in the INL (arrow). **F:** A merged image of **D** and **E**, indicating colocalization of MOR and TH immunolabeling. INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. Dashed lines (**C**) demarcate example focused images used for colocalization analysis (see Methods). Scale bars: 20 μ m.

Figure 4. Localization of DOR immunolabeling in mouse retinal and rat dorsal root ganglion tissues. **A:** 40x confocal image of cryosectioned mouse retina immunolabeled with an anti-DOR antibody (Alomone). Note the puncta in the inner retina with putative somatic labeling in the INL (arrows). **B:** 40x image similar to **A** showing control peptide preadsorption for DOR (Alomone) antibody. **C:** 40x confocal image of rat DRG with DOR+ somas (green). Colabeled with the nuclear marker ToPro3 (red). **D:** 40x image similar **C** showing preadsorption of DOR antibody with control peptide, colabeled with ToPro3 (red). ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. Scale bars: 20µm.

Figure 5.

Multiple inner retinal cell-types including dopaminergic amacrine cells are DOR+. **A:** 40x single-plane confocal image, vertical section of mouse retina showing DOR+ (red; Alomone) somata (arrow). **B:** Image displaying the same region as **A**, immunolabeled for TH (green). **C:** A merged image of **A** and **B**, showing a DOR+ and TH+ amacrine cell in the INL (arrow). **D:** 40x single-plane merged confocal image of vertically sectioned wild-type mouse retina coimmunolabeled for DOR (red; Millipore) and Brn-3a (green). Some Brn-3a+ retinal ganglion cells are MOR+ (arrow). Arrowhead indicating a putative DOR+ soma in the INL. **E:** A 40x single-plane merged confocal image of cryosectioned GAD67-EGFP mouse retina co-immunolabeled for DOR (red; Millipore) and TH (blue). GAD67-EGFP somas are seen in the INL (bright green) and GCL (dim green) with processes in the IPL. A GABAergic (EGFP+) displaced amacrine cell in the GCL is DOR+ (arrow). Some putative DOR+ somas in the GCL are GAD67-EGFP negative (arrowhead). In the INL, a TH+ soma (blue) is DOR+ (asterisks). **F:** Focused view of dopaminergic amacrine cell from **E** showing that the TH+ soma is EGFP- and DOR+. INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. Scale bars: **C**, **D**, and **E**: 20 μ m, **F**: 10 μ m.