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Light regulation of retinal dopamine that is independent of melanopsin phototransduction

M.A. Cameron¹, N. Pozdeyev², A.A. Vugler³, H. Cooper¹, P.M. Iuvone^{2,4}, and R.J. Lucas^{1,†}

¹*Faculty of Life Sciences, University of Manchester, Manchester, United Kingdom*

²*Department of Pharmacology, Emory University School of Medicine, Atlanta, Georgia, USA*

³*Institute of Ophthalmology, University College London, London, United Kingdom*

⁴*Department of Ophthalmology, Emory University School of Medicine, Atlanta, Georgia, USA*

Abstract

Light dependent release of dopamine (DA) in the retina is an important component of light-adaptation mechanisms. Melanopsin-containing inner retinal photoreceptors have been shown to make physical contacts with DA amacrine cells and have been implicated in the regulation of the local retinal environment in both physiological and anatomical studies. Here we determined whether they contribute to photic regulation of DA in the retina as assayed by the ratio of DA with its primary metabolite, DOPAC, and by c-fos induction in tyrosine hydroxylase (TH) labeled DA amacrine cells. Light treatment ($\sim 0.7 \log W/m^2$ for 90min) resulted in a substantial increase in DA release (as revealed by an increase in DOPAC:DA ratio) as well as widespread induction of nuclear c-fos in DA amacrine cells in wild type mice and in mice lacking melanopsin (*Opn4^{-/-}*). Light induced DA release was also retained in mice lacking rod phototransduction (*Gnat1^{-/-}*), although the magnitude of this response was substantially reduced compared to wild-types, as was the incidence of light-dependent nuclear c-fos in DAergic amacrine cells. By contrast, the DAergic system of mice lacking both rods and cones (*rd/rd cl*) showed no detectable light response. Our data suggest that light regulation of DA, a pivotal retinal neuromodulator, originates primarily with rods and cones and that melanopsin is neither necessary nor sufficient for this photoresponse.

Keywords

dopamine; melanopsin; retina; tyrosine hydroxylase; mice; light; amacrine cells; adaptation

Introduction

The influential neuromodulator dopamine (DA) is produced in the vertebrate retina by a subset of specialised interplexiform amacrine cells whose arbours contact the outer plexiform layer and form a plexus in the outer portion of the inner plexiform layer. Its release is strongly light dependent (Iuvone et al., 1978; Parkinson and Rando, 1983, 1983; Umino and Dowling, 1991; Witkovsky et al., 2000) and DA plays a key role in light adaptation in retinal pathways including modulation of coupling between various cell types (Witkovsky, 2004).

The retinal pathways by which light influences DA amacrine cell activity is not clearly understood, but pharmacological analyses have provided some insight. These suggest that activation of DA amacrine cells involves both an excitatory glutamatergic input and disinhibition

† To whom correspondence should be addressed: Email: E-mail: robert.lucas@manchester.ac.uk; tel: +44(161) 275 5050.

due to the loss of a tonic GABAergic signal. The inhibitory input is thought to originate with GABAergic and glycinergic amacrine cells (Kolb et al., 1991; Gustincich et al., 1997; Kolb et al., 1997), whose activity is in turn regulated in a light-dependent manner by cone OFF and rod ON bipolar cells. The excitatory glutamatergic input has been assumed to come from bipolar cells (suggested by Marshak (2001) to be wide field or bistratified ON bipolar cells). However, DA amacrine cell processes are mainly found in the OFF sub-lamina of the IPL making this excitatory glutamatergic input from ON bipolar cells, which stratify in the ON sub-lamina, unlikely. Explanations for this apparent conundrum have hypothesised the existence of ON bipolar cells that stratify paradoxically in the OFF sub-lamina (Hokoc and Mariani, 1987; Marshak, 2001), or the involvement of an acetylcholine amacrine cell interneuron linking ON bipolar terminals to DA amacrine cells (Mariani and Hokoc, 1988).

An alternative origin for photic input to the DA amacrine cells that has not previously been directly examined is the melanopsin class of intrinsically photosensitive retinal ganglion cells (ipRGCs). These ganglion cell photoreceptors are thought to be optimised for the sort of sustained irradiance-coding task that defines an important aspect of the DA amacrine cell light response and growing evidence supports their involvement. Firstly, there is a close physical association between their dendrites and those of DA amacrine cells (Vugler et al., 2007). Secondly, a precedent for intra-retinal export of their light information exists in the form of gap-junction coupling to neighbouring cells in the ganglion cell layer (Sekaran et al., 2003). Thirdly, studies of circadian and light adaptation of human and mouse ERGs (Hankins and Lucas, 2002; Barnard et al., 2006) have implicated ipRGCs in contributing to the sorts of network reorganisation that DA has been associated with. Finally, a recent electrophysiological characterisation of light responses in DAergic amacrine cells has revealed evidence that melanopsin drives a sustained depolarisation in a subset of these cells (Zhang et al., 2008).

Here we address the role of melanopsin in DA amacrine cell regulation by comparing light dependent DA release and nuclear c-fos accumulation in wild type mice and transgenic animals lacking either melanopsin (*Opn4*^{-/-}), rod phototransduction (*Gnat1*^{-/-}) or both rod and cone photoreceptors (*rd/rd cl*). Our data reveal that at least the major component of light input to both these aspects of the DAergic light response originates with classical rod and cone photoreception.

Methods

Animals

Mice were bred and housed in the University of Manchester. All procedures were conducted according to the UK Animals (Scientific Procedures) Act of 1986. C3H^{F+/+} mice were used as congenic wild-type controls for *rd/rd cl* mice (Lucas et al., 1999). *Opn4*^{-/-} and *Gnat1*^{-/-} mice were on a C57BL/6 and 129sv mixed strain background and wild-types on the same mixed strain background used as controls. Mice (70-120 days of age) were housed under a stable 12 hr light (white fluorescent ~0.7 log W/m² at cage floor):12 hr dark cycle with food and water *ad libitum* for > 1 week prior to tissue collection.

Animals were killed and retinas rapidly collected for immunohistochemistry (IHC) and HPLC under dim red light (<0.1 W/m²; >650nm) at either the mid point of the subjective night (CT18) or the following subjective day (CT6) under conditions of constant darkness (that is after being in darkness for 6 or 18hrs respectively). Light treated groups were exposed to 90 min white fluorescent light (~ 0.7 log W/m² at cage floor) in their home cage immediately prior to tissue collection.

Immunohistochemistry

The left eye from each animal was fixed in 4% PFA overnight, cryoprotected in 30% sucrose and then frozen rapidly in OCT embedding matrix (Raymond A Lamb Ltd., UK). Tissue sections (10µm) were collected from the central retina and on poly-lysine coated slides (VWR International). These sections were then blocked with 5% normal donkey serum (Jackson ImmunoResearch) and labeled concurrently with sheep anti-tyrosine hydroxylase (Chemicon AB1542, 1:1000 dilution (a pan-TH antibody recognizing all phosphorylation states)) and rabbit anti-c-fos (Calbiochem PC38, at 1:5000 dilution) antibodies. Secondary FITC anti-rabbit and TRITC anti-sheep antibodies (preadsorbed to multiple species and especially designed for multiple labelling, Jackson ImmunoResearch; catalogue no: 713-095-147 (FITC), 711-025-152 (TRITC)) were then applied. All antibody incubations were performed with 0.1M PBS containing 0.3% triton X-100 (BDH) and sections were washed extensively in PBS between application of primary and secondary antibodies. Slides were cover-slipped with Vectashield containing DAPI to stain nuclei (Vector labs). Wholemound retinæ were also prepared and processed as above, although in this case primary antibody solutions contained 3% triton to aid antibody penetration (Vugler et al., 2007). Nuclear c-fos expression in 10 TH-labelled cells from each animal were analysed at random and assessed by eye for nuclear c-fos immunoreactivity. These cells were given a score of positive or negative for nuclear c-fos and the percentage per animal calculated. Determining c-fos positive cells was straightforward for most genotypes as a strong FITC fluorescence signal (approx 8× FITC signal of the retina; quantified by Leica software) was observed colocalised with DAPI within at least some TH labelled cells under light treated conditions. In order to detect any potentially weaker nuclear signal, contrast was then increased to maximum (allowing any signal >3× mean energy of background to be detected). Any TH cells displaying nuclear c-fos under these conditions were also scored positive, although in practice this never occurred.

Dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) analysis by high-performance liquid chromatography with coulometric detection

Levels of dopamine and DOPAC in mouse retina were determined by ion-pair reversed-phase high-performance liquid chromatography (HPLC) with coulometric detection (guard cell at 0.4V, and coulometric analytical cell at 0.3V) as described elsewhere (Pozdeyev et al., 2008). Retinas were homogenized in 50µl of 0.2 N HClO₄ containing 0.01% of sodium metabisulfite and 25ng/ml of internal standard 3,4-dihydroxybenzylamine hydrobromide. After centrifugation at 15,000g for 10 minutes, a 40µl aliquot of supernatant was analyzed. The separation was performed on an Ultrasphere ODS 250 × 4.6 mm column, 5µm (Beckman Coulter, USA) with a mobile phase containing 0.1M sodium phosphate, 0.1mM EDTA, 0.35mM sodium octyl sulfate, 5.5 % acetonitrile (vol/vol), pH 2.7. External standards of dopamine and DOPAC were analyzed in each experiment.

Results

Rodless+coneless mice

We first investigated photic control of DAergic amacrine cells by exploring nuclear c-fos accumulation in TH positive cells of wild type and congenic *rd/rd cl* mice in response to 90 min bright light exposure. In order to account for any potential time of day effects, these experiments were undertaken both at subjective midday and midnight. In both genotypes, and at both times of day, the soma of DA amacrine cells (identified by immunoreactivity for tyrosine hydroxylase (TH)) appeared sparsely distributed in the inner portion of the INL and their dendrites as a plexus in the outer IPL. In the dark, cytosolic c-fos labelling of TH labelled cells was observed in all groups. However, only a very few DAergic cells showed nuclear c-Fos labelling in the dark, which was diffuse in nature (Figure 1 A, E). Light exposure caused had a dramatic effect on wild types at both times of day, inducing nuclear c-fos accumulation

(Figure 1 B, F), suggesting either nuclear translocation of cytosolic c-fos (as previously reported (Roux et al. 1990)) and/or de novo synthesis of this protein in response to activation of these cells. When quantified, it was revealed that > 90% of TH cells in wild-type mice display nuclear c-fos localization when stimulated by light (Figure 1 I; 1-way ANOVA; $F(3,12)=381.4$; $p<0.0001$). In contrast, however, the proportion of TH cells expressing nuclear c-Fos in *rd/rd cl* mice was unaffected by light exposure at any time of day (Figure 1 J; 1-way ANOVA; $F(3,15)=0.542$; $p>0.05$). Indeed, > 96% of TH cells did not show nuclear c-fos immunoreactivity above baseline levels.

We next explored light induced DA release by measuring the retinal DOPAC:DA ratio by HPLC. DOPAC is the major metabolite of DA in the mammalian retina and is produced primarily after the re-uptake of dopamine to the DAergic cell after release (Cooper JR, 1986). The retinal DOPAC:DA ratio has therefore been used as a good indicator of how much DA has been released and subsequently transported back into the DA amacrine cells (Witkovsky, 2004). Wild-types showed a significant increase in this ratio following light treatment (Figure 2 A; 1-way ANOVA; $F(3,24)=25.23$; $p<0.0001$). The magnitude of this effect was larger at subjective midnight. This may reflect a circadian influence on DA metabolism, or could simply reflect that the animals shield their eyes from the light during their inactive phase. Unlike wild types, we observed no significant change in the DOPAC:DA ratio in *rd/rd cl* mice in response to light (Figure 2 B; 1-way ANOVA; $F(3,12)=0.270$; $p>0.05$).

Melanopsin-deficient mice

As a further test of the hypothesis that ipRGCs provide photic regulation of the DAergic system we next set out to determine whether this response was impaired in mice lacking melanopsin. Once again, TH IHC revealed DAergic amacrine cells with the expected morphology and density in both *Opn4^{-/-}* and wild type littermates. In the dark, some cytosolic, but little nuclear c-fos was observed in these cells in both genotypes at both CT6 and CT18. Upon light exposure all treatment groups responded with a substantial increase in the presence of c-fos in the nucleus of TH positive cells (Figure 3 A-D). Quantification revealed a significant increase in the percentage of cells positive for nuclear c-fos following light stimulation in both genotypes (Figure 3 G&H; 1-way ANOVA; $F(3,13)=129.4$; $p<0.0001$ for wild-type; $F(3,13)=12.59$; $p<0.001$ for *Opn4^{-/-}*). There was no detectable difference in the degree of response between the genotypes (2-way ANOVA; $F(1,26)=2.115$; $p>0.05$; no significant interaction).

The 90 min light stimulus also induced a substantial increase in the DOPAC:DA ratio in both wild-type and *Opn4^{-/-}* mice at both times of day (Figure 4 A&B; 1-way ANOVA; $F(3,22)=22.45$ for wt; $F(3,21)=37.76$; $p<0.0001$). In common with the results for C3H wild type mice (Figure 2 A) the response to light was larger at CT18. However, there was no significant difference in the response between the two genotypes (2-way ANOVA; $F(1,43)=1.391$; $p>0.05$; no significant interaction).

Mice lacking rod phototransduction

The inference from the *rd/rd cl* and *Opn4^{-/-}* experiments is that rod and/or cone photoreceptors are responsible for the greater part of the DAergic light response. In view of the substantial interconnectivity between rod and cone pathways in the retina (Raviola and Gilula, 1973; Sharpe and Stockman, 1999) it is most likely that both influence DAergic cells. Nevertheless, in order to determine whether this was so, we assessed light responses in *Gnat1^{-/-}* mice that lack rod transducin, an essential component of rod phototransduction (Calvert et al., 2000). We found that these animals also show a significant light induced increase in the DOPAC:DA ratio (Figure 4 C; 1-way ANOVA; $F(3,16)=11.30$; $p<0.001$), although its magnitude was substantially reduced compared to that of wild types. Consistent with this finding, the incidence of light induced nuclear c-fos in DAergic amacrine cells was greatly reduced in this genotype. Thus,

while retinal whole mounts revealed occasional c-fos positive DA amacrine (often appearing in patches despite the fact that c-fos staining in non-DAergic cells was uniform across the retina), these were sufficiently rare not to occur in sections taken from the central retina and used for quantification of this response (Figure 3 E, F, I; 1-way ANOVA; $F(3,11)=0.896$; $p>0.05$). This suggests that while cones alone can drive some DA release, the combined action of both rods and cones is responsible for the wild type response.

Discussion

In common with previous work in mice and many other vertebrate species, our data reveal a robust light induced activation of DA amacrine cells as assessed both by nuclear c-fos accumulation and DA release. These light responses were neither present in mice lacking rods and cones (*rd/rd cl*), nor noticeably impaired in those lacking melanopsin (*Opn4^{-/-}*). Consequently, our data suggest that the retinal DAergic system relies primarily upon rods and cones for its photosensitivity.

Previous attempts to assess the importance of classical photoreceptors for DA release using retinally degenerate models have produced contradictory results (Morgan and Kamp, 1980; Frucht et al., 1982; Nir and Iuvone, 1994). Our data suggest that this variability can be attributed to differences in the degree of degeneration. The *rd/rd cl* model used here was originally created to overcome the problem that even the most severe forms of outer retinal degeneration are generally incomplete. *rd/rd cl* animals are homozygous for the *rd1* mutation which abolishes rod phototransduction and results in outer retinal degeneration. However, since significant numbers of functional cones survive in *rd/rd* animals (even into old age) (García-Fernández et al., 1995; Jimenez et al., 1996; LaVail et al., 1997), the *rd/rd cl* mice carry a transgene targeting diphtheria toxin expression to these photoreceptors (Soucy et al., 1998). The result is an animal that lacks detectable rods and cones (Lucas et al., 1999). The inner retina of these mice, although undoubtedly subject to remodelling, is intact at the age used in the present study and the amacrine and ganglion cells (including ipRGCs) remain physiologically active (Sekaran et al., 2003; Marc et al., 2007). Consequently, the lack of light induced DA release in these animals represents compelling evidence that melanopsin photoreceptors alone cannot drive this response to a significant extent.

Opn4^{-/-} mice, conversely, offer a good opportunity to test the hypothesis that melanopsin phototransduction is necessary for photic regulation of DA release. Rod and cone pathways are physiologically normal in these mice (Fu et al., 2005; Barnard et al., 2006) and our data reveal that they alone are capable of driving full amplitude DA light responses. However, as ipRGCs remain structurally intact in *Opn4^{-/-}* mice our findings do not exclude the possibility that some aspect of the rod/cone input to DA amacrine is routed via these cells (Guler et al., 2008).

Electrophysiological recordings have suggested three distinct light response phenotypes for mouse TH-expressing amacrine cells: ~38% (classified as ON-transient) respond with a rapid burst of spikes elicited near the onset of the light stimulus, followed by a decrease in spike frequency, for the duration of the stimulation; ~22% (classified as ON-sustained) respond with a light-evoked increase in action potential frequency which was maintained throughout the duration of the light pulse; while the remaining ~40% fail to show a light response (Zhang et al., 2007). In wild type mice we reliably observed nuclear c-fos accumulation in >90% of TH positive cells following light exposure. These cells were identified morphologically as type 1 TH-expressing cells (characterised by large somas and processes in the upper lamina of the IPL) and it is likely that the antibody we used only recognises type 1 TH-expressing cells and not the Type 2, possibly noradrenergic, TH cells (Gustincich et al., 1997; Zhang et al., 2004). Nevertheless, the almost universal c-fos response that we observed in wild types is surprising

and does not support the hypothesis that 40% of type I TH cells are light-independent (Zhang et al., 2007).

Of the remaining response phenotypes, that of ON-sustained most closely resembles the light response properties (slow and sustained) of ipRGCs. While preparing this manuscript another study presenting evidence that melanopsin can drive a sustained electrophysiological response in this group of TH positive cells was published (Zhang et al., 2008). Those findings are not necessarily inconsistent with our data, as neuronal activation need not induce either DA release or c-fos induction (Gonon, 1988; Machado et al., 2008). On the other hand, it is worth pointing out that the genetic approach used by Zhang et al (2008) to identify DAergic cells for physiological recoding also labels type 2 TH positive amacrine cells (Zhang et al (2004) that may be noradrenergic/adrenergic (Hadjiconstantinou and Neff, 1984; Gustincich et al., 1997). The authors exclude the possibility that their recorded light responses are actually from these cells on the basis of their morphology, but this distinction can be hard to draw with certainty.

Zhang et al (2008) also report light-dependent induction of c-fos in 20% of TH positive cells in *rd/rd* mice. Our failure to observe nuclear c-fos in DAergic amacrine cells in light treated *rd/rd cl* mice appears to directly contradict that finding. There are a number of potential explanations for the discrepancy:

1. This could reflect the fact that different primary antibodies were used for identifying TH. Some TH antibodies also label the other non-DAergic type 2 catecholaminergic amacrine cells (Mariani and Hokoc, 1988), whereas others do not pick up these type 2 TH positive amacrine cells (Gustincich et al., 1997; Zhang et al., 2004). As type 2 TH positive cells have been shown to be less strongly immunoreactive for TH than DAergic cells, this difference in labelling presumably reflects differences in the degree of antibody reactivity. This raises the possibility that the c-fos induction reported by Zhang et al (2008) may arise in these non-DAergic cells not revealed by our antibody.
2. As Zhang et al (2008) used older animals than those employed here the discrepancy could reflect progressive remodelling of inputs to DAergic cells. *rd/rd* mice exhibit significant remodelling of retinal ganglion cells well into adulthood (Wang et al., 2000), and Nir and Iuvone (1994) have previously reported age dependent increases in DAergic photosensitivity in a similar model of retinal degeneration (the *rds* mouse). Robust light-evoked stimulation of DOPAC levels in retinas of young (1 mo. old) wild type controls was reported but no significant effect of light was observed in age-matched *rds* mice. Surprisingly, one year old *rds* mice displayed a small but significant light-evoked elevation in dopamine metabolism.
3. It also is possible that the degree of c-fos induction in DAergic cells is below the detection threshold of our experiments. Zhang et al (2008) make no comment on the amplitude of c-fos induction in their experiments and we can not entirely exclude c-fos induction in ours, although such a response would have to be very small compared to that elicited by rods and cones.
4. Zhang et al (2008) do not distinguish between nuclear and cytoplasmic c-fos. We find occasional cytoplasmic c-fos in TH positive cells in the dark, but nuclear c-fos only following light-activation. This suggests that, as part of the AP-1 transcription factor complex, it is the nuclear location of this protein that is activity dependent.

Whether or not DAergic cells receive excitatory input from ipRGCs, our HPLC analysis of retinal DA in *rd/rd cl* and *Opn4^{-/-}* mice argue that this pathway does not make a significant contribution to regulating tissue wide DA release. This does not in itself contradict Zhang et al's evidence for ipRGC-dependent electrophysiological activation of DAergic cells as DA

release can depend critically upon the nature of spiking with the sorts of slow sustained activation typically associated with ipRGC activity relatively ineffective (Gonon, 1988). Thus, it remains possible that ipRGC activation of DA amacrine serves some purpose other than regulating tissue-wide DA concentrations. This might be entraining local circadian clocks, a function previously suggested for ipRGCs (Barnard et al., 2006). Alternatively, ipRGCs may drive a modest DA release, below the sensitivity of our assays, which could have local autocrine/paracrine or neurotransmitter functions. Nonetheless, on the basis of our findings the major neuromodulatory role of DA in retinal light adaptation must now be considered to be independent of inner retinal photoreception.

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Abbreviations

DA	dopamine
DOPAC	dihydroxyphenylacetic acid
Gnat1	G protein alpha transducing activity polypeptide 1
HPLC	high performance liquid chromatography
IHC	immunohistochemistry
ipRGC	intrinsically photoresponsive retinal ganglion cell
<i>Opn4</i>	melanopsin
<i>rd/rd cl</i>	<i>rodless + coneless</i>
TH	tyrosine hydroxylase

Figure 1. *rd/rd cl* mice lack light-dependent c-fos induction in dopaminergic amacrine cells

A-H, Representative images of retinal sections from wild-type C3H $f^{+/+}$ (A-D) and congenic rodless+coneless (*rd/rd cl*) mice (E-H) at subjective midday (CT6; A, B, E, F) and midnight (CT18; C, D, G, H) in the dark (A, C, E, G) or following a 90min light pulse (B, D, F, H), stained by IHC for c-fos (red) and TH (green). These images reveal light induced nuclear c-fos in wild-type, but not *rd/rd cl* mice. Blue staining is DAPI; scale bar 10µm. **I&J**, This result is confirmed by quantification of the percent TH expressing cells with nuclear c-fos localisation in wild-type (I) and *rd/rd cl* mice (J) for each light condition (mean±SEM; n=4 for all conditions). Light caused a significant increase in this percentage in wild-types at both times of day (**p<0.001 for CT6 dark vs CT6+light and CT18 dark vs CT18+light; Bonferroni

post-test) but not in *rd/rd cl* mice. **K-N**, Images taken from the dorsal retina of whole-mounted preparations from wild-type (K-L) and *rd/rd cl* mice (M-N) stained for TH (green) and c-fos (red), confirm the presence of nuclear c-fos in wild types following a 90 min light pulse (L) but not in wild types in the dark (K) or in *rd/rd cl* under either condition. Scale bar 50µm.

Figure 2. *rd/rd cl* mice lack light induced dopamine release in the retina

A. Light caused substantial DA release (reflected in an increase in the DOPAC:DA ratio) in wild-type C3H $f^{+/+}$ mice at both CT6 and CT18 (* $p < 0.05$ CT6 dark vs CT6+light; *** $p < 0.001$ CT18 dark vs CT18+light; Bonferroni post-test; mean $\pm$ SEM; $n = 6$). **B.** By contrast there was no indication of significant light-dependent DA release in *rd/rd cl* mice (mean $\pm$ SEM CT6 dark: $n = 3$, CT6 light: $n = 4$, CT18 dark: $n = 6$ CT18 light: $n = 3$).

Figure 3. Normal light-dependent c-fos induction is retained in *Opn4*^{-/-} but not *Gnat1*^{-/-} mice

A-F, Representative images of retinal sections collected at CT18 (subjective midnight) in the dark (**A**, **C**, **E**) or following a 90min light pulse (**B**, **D**, **F**) and stained by IHC for c-Fos (red) and TH (green) reveal light induced nuclear c-fos in wild-type (**A** & **B**) and *Opn4*^{-/-} (**C** & **D**) but not in *Gnat1*^{-/-} (**E** & **F**) mice. Blue staining is DAPI; scale bar 10μm. **G-I**, The percentage of TH expressing cells showing nuclear c-fos signal (mean±SEM; n=4 for all conditions) was significantly enhanced following light exposure at both CT6 and CT18 in wild-type (**G**) and *Opn4*^{-/-} (**H**) (***p<0.001 CT6 dark vs CT6+light; **p<0.01 CT18 dark vs CT18+light; Bonferroni post-test) but not in *Gnat1*^{-/-} mice (**I**).

Figure 4. Light induced dopamine release relies on rods and cones

90 min light exposure caused substantial DA release (reflected in an increase in the DOPAC:DA ratio; mean±SEM; n=5-6) in both *Opn4*^{-/-} (A) and wild-type (B) mice and to a lesser extent *Gnat1*^{-/-} mice (C) at both CT6 and CT18 (*p<0.05 CT6 dark vs CT6+light in wild-type and *Gnat1*^{-/-} mice; **p<0.01 CT6 dark vs CT6+light in *Opn4*^{-/-} mice; ***p<0.001 CT18 dark vs CT18+light in all genotypes; Bonferroni post-tests).