Letter to the Editor

Slow light response kinetics in rods points toward a perturbation of the normal cellular milieu

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Running title: Rod photoreceptors and perforated patch recordings

In a recent paper Jin et al. (2015) investigate electrical coupling between rod photoreceptors in the mouse retina and its circadian regulation. To obtain their data the authors use perforated patch clamp recordings from rod inner segments in whole isolated retinas.

Due to their small size and complex biochemical machinery, mouse photoreceptors (and thus, in all likelihood, mammalian photoreceptors in general) are easily perturbed by the very same electrophysiological technique that is used to record from them: our lab has recently voiced this issue showing that perforated patch recordings of mouse rods and cones can affect membrane potentials and flash response kinetics (Cangiano et al., 2012), as well as rod-cone coupling (Asteriti et al., 2014). Jin and colleagues are aware and rightfully concerned of these potential problems, as from their statement: "*A recent study has reported that rod-cone coupling increases spontaneously after obtaining intracellular access in mouse cones and that rundown of kinetics with time are often observed in patched rods (Asteriti et al. 2014). In our conditions, we observed a run down in rod kinetics following intracellular access in less than 10% of the rods. This phenomenon typically developed within minutes, and the data obtained under these conditions were discarded. Differences between the technique used by Asteriti et al. and ours include a different perforating agent (Amphotericin-B vs*

β-escin), the presence of ATP in our pipette solution and its absence in theirs, and a higher resistance of the pipettes we used (15-20 MΩ vs 6-9 MΩ).". Here the reader is led to surmise that the light response kinetics of the rods presented in Jin et al. (2015) were physiological and, therefore, that their recordings did not affect the photoreceptors. We argue that their data actually provide support for the opposite conclusion. However, it must be first clarified that a rundown of rod response kinetics was indeed originally reported by us (Cangiano et al., 2012), but that we later successfully circumvented this problem by recording from rods with a loose seal technique (Asteriti et al., 2014). Moreover, contrary to what stated by Jin et al., we found that, with patch recording, rod-cone coupling increases spontaneously even with ATP/GTP in the pipette.

Jin et al. provide only one type of kinetics data that can be used for comparing to other studies, namely the time-to-peak (TTP) of the single photon response. It is important to note that they report separate 'latency' and 'time-to-peak' components that must be summed together to obtain the TTP normally used in the literature (from the flash to the peak of the response). In fact, the latency component is equally biological in origin, as can be inferred from: (*i*) the negligible delay introduced by their low-pass Bessel filtering at 1 kHz, and (*ii*) the dramatic reduction in latency occurring with bright flashes (Fig. 4F). It follows that their true TTP at 32°C, obtained using their tabulated data, was 410 and 458 ms (mean day and night values, respectively; Tables 3-4). These values are further supported in the plots of their figure 4F/G, presenting a larger rod sample. Even considering only the smaller value (daytime), which in the words of the authors reflects rods "essentially in a decoupled state or electrically isolated from other photoreceptors", it is very large. Much larger, for instance, than: (*i*) our estimates, *using perforated patch clamp data*, of unperturbed mouse rod kinetics of 256 ms at 24°C ($p<0.01$ relative to Jin et al.; Mann-Whitney test) and 147 ms at 36°C ($p<0.001$) (Cangiano et al., 2012); (*ii*) our measurements, made with a loose seal technique known not to affect mouse rod kinetics, of 258 ms at 24°C (Asteriti et al., 2014); (*iii*) *in vitro* electroretinographic (ERG) measurements of rat rods of 280 ms at 28°C and 150 ms at 36°C (Nymark et al., 2005); (*iv*) published estimates of mouse and other mammalian

rods recorded with patch clamp, suction electrodes or ERG near body temperature (i.e. only a few degrees above the 32°C used by Jin et al.) covering the range 104–218 ms (several references in Cangiano et al., 2012)(Sakurai et al., 2011; Sarfare et al., 2014).

It further surprised us the statement of Jin et al. (2015) that the "*kinetics of the current and voltage responses to light compared favourably with those measured with suction (Field and Rieke, 2002a) and patch-clamp (Cangiano et al. 2012) electrodes in mouse retinal slices*": if the authors meant to cite Field & Rieke (2002b), since it is on mouse, the only kinetics data to be found must be extracted from the record of the dim flash response of one single rod, with a rather short TTP of ~200 ms (Fig. 1B); if, instead, the authors really wanted to cite Field & Rieke (2002a), this study is on guinea pig and macaque, and a similarly short TTP of ~200 ms for two rods must be extracted from figures. As regards the citation to Cangiano et al. (2012), this is the paper describing the slowing effect of perforated patch recordings on rod response kinetics!

We can only think of two explanations for the slow rod responses presented by Jin and colleagues: (*i*) their recordings did perturb the cellular milieu of rods, perhaps during the initial ~10 min required for patch perforation by β-escin; (*ii*) such perturbation was already present in rods prior to patching. Either possibility raises the serious concern that rod-rod coupling, the main focus of their study, may have also been affected in their recordings — further compounded by evidence that rod-cone coupling is regulated on the rod side of the junctional contacts (Asteriti et al., 2014). This would significantly complicate the interpretation of their findings, which rely on estimating the physiological strength of rod-rod coupling in the day and night.

In a first scenario, such a perturbation affected equally all recordings and, as a result, rod-rod coupling may have simply been shifted from its physiological working range, enhancing or attenuating the amplitude of circadian modulation. In a second scenario, the entity of the perturbation itself changed between day and night, either because of unintentional but systematic differences in the experimental conditions (circadian time may affect both the environment and the operator), or due to a circadian variation in the susceptibility of rods to the perturbation. In the last scenario, the circadian modulation of rod-rod coupling reported by Jin et al. (2015) could have been a

byproduct of the circadian-dependence of the perturbation. While this is admittedly a less likely scenario, suggestive evidence for it is the magnitude of the changes in both the dim and the saturating flash responses at night compared to the day: up to twofold slower kinetics (Fig. 4A-H, Tables 3-4) and around halved amplitude (Fig. 3, Tables 1-2). Given the evidence that we present above that in these experiments the rods were indeed perturbed, these changes in kinetics and amplitude could directly reflect differing degrees of such rod perturbation. Jin et al. report that the dopaminergic agonists/antagonists quinpirole and spiperone have opposite effects on rod kinetics and amplitude, which would argue against this possibility. Nevertheless, the quality of the photovoltage records that should be representative of the data upon which their conclusion is drawn, does not appear sufficient for an unambiguous demonstration (Figs. 8 Aa-Ca, 9 A, 10 Aa-Ba; compressed time scales, low signal-to-noise ratios, atypical waveforms after saturating flashes — highly variable even in the same rod).

It is important to emphasise that while patch recordings can affect the functional state of mammalian photoreceptors, this does not imply that physiologically-relevant conclusions cannot be made using this technique: one must realise its limitations and plan for appropriate controls, timing of the recordings or devise alternative approaches. This has been our goal both when measuring the single photon response (Cangiano et al., 2012) and when demonstrating rod-cone coupling (Asteriti et al., 2014).

References

Asteriti S, Gargini C & Cangiano L. (2014). Mouse rods signal through gap junctions with cones. Elife 3, e01386.

Cangiano L, Asteriti S, Cervetto L & Gargini C. (2012). The photovoltage of rods and cones in the dark-adapted mouse retina. The Journal of physiology 590, 3841-3855.

Field GD & Rieke F. (2002a). Mechanisms regulating variability of the single photon responses of mammalian rod photoreceptors. Neuron 35, 733-747.

Field GD & Rieke F. (2002b). Nonlinear signal transfer from mouse rods to bipolar cells and implications for visual sensitivity. Neuron 34, 773-785.

Jin NG, Chuang AZ, Masson PJ & Ribelayga CP. (2015). Rod electrical coupling is controlled by a circadian clock and dopamine in mouse retina. The Journal of physiology Accepted Article.

Nymark S, Heikkinen H, Haldin C, Donner K & Koskelainen A. (2005). Light responses and light adaptation in rat retinal rods at different temperatures. The Journal of physiology 567, 923-938.

Sakurai K, Young JE, Kefalov VJ & Khani SC. (2011). Variation in rhodopsin kinase expression alters the dim flash response shut off and the light adaptation in rod photoreceptors. Investigative ophthalmology & visual science 52, 6793-6800.

Sarfare S, McKeown AS, Messinger J, Rubin G, Wei H, Kraft TW & Pittler SJ. (2014). Overexpression of rod photoreceptor glutamic acid rich protein 2 (GARP2) increases gain and slows recovery in mouse retina. Cell communication and signaling : CCS 12, 67.

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