Dear PCI reviewers and recommenders,

We thank you for the time you took to review our work and for your feedback. A point-by-point response to all comments is attached in the following.

Reviewer #1:

The preprint examines how immediate early gene expression (IEG) in the mouse primary visual cortex relates to neuronal activity and is influenced by visual experience and visuomotor coupling. IEGs are widely used as a proxy for neuronal activity or plasticity but the exact circumstances that induce IEG expression in vivo remain unclear. The preprint helps fill this important gap. The results suggest that neurons expressing different IEGs show different levels of recruitment by top-down vs bottom-up inputs. Activity of EGR1-expressing neurons is correlated with running, while that of Arc-expressing neurons is correlated with visual flow.

The experiments appear well executed and the analyses largely appropriate, although some improvements are suggested below. The description of the methods, especially those pertaining to data analysis, is very brief and it is sometimes difficult to figure out how exactly the metrics presented in the figures were derived. Some specific examples are mentioned in my comments below.

1. DeltaF/F calculations throughout the preprint do not appear to include neuropil correction. Correcting for neuropil contamination is now a routine step in the analysis of two-photon imaging data. Lack of neuropil correction may obscure some of the differences between IEG-high and IEG-low cells.

This is correct, we do not apply any neuropil correction. Like with any these corrections, or spike inferences, this should never be done as a default. All corrections introduce new artifacts in the data. The idea of neuropil correction originated with calcium recordings using synthetics dyes (like OGB). In these recordings, if the experimenter did not wait long enough after dye injection, extracellular dye could remain in the tissue interfering with the measurement of intracellular calcium activity. Extracellular dye signal confounded cellular responses as the signal tended to anticorrelated with cellular responses. Simplifying neuropil correction in these experiments was the fact that the dye, once taken up by the cell, typically accumulated in the nuclei. With GCaMP recordings, neuropil corrections were initially no longer applied as all GCaMP was now actually intracellular. These corrections were later "rediscovered" for reasons that are not entirely clear to me. With genetically encoded calcium indicators none of the signal is extracellular and the "neuropil" signal, on average, is just a mean population signal. Thus, the correction, even if properly applied subtracts a fraction of the population mean from the activity of individual neurons. Because the subtraction parameter is never actually calibrated by combined calcium and electrophysiological recordings, the experimenter now has an additional parameter to tune results to a desired outcome. Any form of correlation analysis, for example, that is based on data that was "neuropil subtracted" should probably be treated with an extra grain of salt because of this extra parameter the experimenter has at their disposal to "tune" the results. A neuropil correction decorrelates the responses of individual neurons away from the population average. Applying such corrections without calibration seems ill advised.

38 **The rediscovery of neuropil corrections coincided with the advent of large field of view calcium**
39 **recordings. A contamination of the signal by surrounding tissue, as well as movement artifacts, are**
40 **primarily a concern when doing two-photon imaging at a low resolution. Here pixels per cell is the**
41 **relevant dimension. With fields of view in the 1 mm by 1 mm range and resolution of a few hundred**
42 **pixels, this corresponds to cell diameters somewhere on the order of a few pixels. With individual**
43 **neurons resolved at such low resolutions, both blurring, and movement artifacts become more of a**
44 **concern. It is not difficult to do calcium imaging with a large field of view, recording thousands of**
45 **neurons simultaneously – it just comes at a substantial cost in data quality. For this reason, all of our**
46 **recordings are done at high spatial resolution (cells typically 20 pixels in diameter – i.e. 300 or so**
47 **samples per neuron) with fields of view of only 200-300 µm and a few hundred cells per recording.**

48 **Thus, with no calibration of correction parameters to go on, and given that our data are acquired at**
49 **relatively high resolution, it would be a bad idea to apply any form of neuropil correction.**

50 *2. The analysis in Figure 1 examines the effects of transient visual stimulation on IEG expression. As far*
51 *as I understand, panels E-J correlate IEG expression to z-scored fluorescence at time 0 without correcting*
52 *for baseline activity. This explains why the correlations in panels E-G appear acausal. IEG expression*
53 *shows some correlation with neural activity during visual stimulation in the future, presumably because*
54 *future activity is correlated with baseline activity in the dark. As the result, correlations in panels E-G*
55 *reflect the effects of both baseline and visually evoked activity. It might be informative to look at the*
56 *correlation of responses during visual stimulation, while controlling for baseline activity, either by*
57 *subtracting it or using partial correlation.*

58 **This may partially be a misunderstanding. By 'acausal' we assume the reviewer means IEG increases**
59 **preceding activity increases. Positive numbers in Figures 1E-J indicated an IEG measurement that**
60 **follows the activity measurement (e.g. +3.5 hours means the IEG was measured 3.5 hours after the**
61 **activity). Hence the IEG expression is not acausal in relation to the calcium activity measurement.**

62 **By "baseline subtraction" we assume the reviewer means baseline subtraction of calcium activity. If**
63 **so, this is not entirely correct. Calcium activity is baseline corrected by virtue of the dF/F_0**
64 **normalization. F_0 is the median fluorescence, and our estimate of "baseline". This is standard**
65 **procedure for calcium imaging experiments without trial structure.**

66 **We have rephrased the axis labels as well as the legends to make this clearer.**

67 *3. Figure 2B reports "a significant negative slope" of calcium activity following visual stimulation. It is*
68 *not clear what individual samples are included in the regression analysis. Single time points across mice*
69 *or averages? In any case, as time points are not independent, the computed p-value may not be*
70 *meaningful and it might be more appropriate to only report the paired comparisons between conditions.*

71 **We have expanded on the description of the analysis in the methods section to make this clearer.**

72 **A linear trend analysis is a relatively standard way of analyzing this type of data. It is correct that the**
73 **samples are not independent, but hence the trend analysis. The aim of the linear trend analysis is to**
74 **test how well the change in activity is explained by a linear function of time. Please note, we also**
75 **compare conditions 1 and 3 directly.**

76 *4. Figure 3 presents average responses of IEG expressing neurons to running onset and grating*
77 *presentation. It would be useful to also show the histograms of response magnitude for each IEG and*
78 *condition to help readers assess the heterogeneity of responses within each IEG population.*

79 **Thank you for catching this, the description of these results was badly phrased. Responses are**
80 **different on average - we have rephrased this. While differences are apparent on average, the**
81 **response distributions clearly overlap, hence the relatively large SEMs. The full distributions are**
82 **similar to those already shown in Figure 4A, hence we don't think showing them again here would add**
83 **much.**

84 *5. Figure 4A ostensibly shows one of the punchlines of the preprint but the scatter plot is very difficult*
85 *to read. The data may be more clearly presented as a circular histogram for each IEG (as in Roth, M et*
86 *al., Nat Neurosci 19, 299–307 (2016)).*

87 **We thank the reviewer for the suggestion, but the display of the data is matched to the way we**
88 **display these types of data in other work (see e.g. (Attinger et al., 2017; Widmer et al., 2022)) and we**
89 **would prefer to keep it this way. The inset showing the means, that we use to quantify the effects,**
90 **should make the comparison straightforward. We did try circular historgrams, but the main problem**
91 **with this representation is that it does not capture the the distance from 0. As in most datasets**
92 **running correlation is slightly positive on average, this moves most data into the same direction in a**
93 **circular histogram even if they are quite well separated in a 2d representation. Roth et al. partially**
94 **address this problem by excluding data close to zero in their circular histogram representation.**

95 ***Reviewer 2:***

96 *Mahringer et al. explore how the expression of different immediate early genes (IEGs, specifically Arc, c-*
97 *fos and EGR1) in layer 2/3 of the primary visual cortex (V1) are correlated with visual and visuomotor*
98 *experiences, which might indicate that different forms of plasticity are associated with distinct input*
99 *pathways.*

100 *The Authors used transgenic mouse lines that express GFP under the respective promoters. Arc and c-fos*
101 *were fused to GFP and expressed under the respective promoters, whereas the EGR1 promoter was*
102 *driving GFP expression. Additionally, the jRGEKO red calcium indicator was expressed in layer 2/3*
103 *populations with the help of an AAV virus. Regarding the experimental protocols, the Authors measured*
104 *behavior through quantification of movement on a styrofoam ball, exposed mice to visual gratings and*
105 *also combined the two in virtual reality in order to assess normal visuo-motor coupling, and also under*
106 *perturbations in order to quantify neuronal coding for mismatch signals.*

107 *They show that neurons strongly activated by visuomotor input are more likely to express EGR1, while*
108 *visual input driven neurons show a strong expression of Arc, two IEGs previously shown to participate in*
109 *pathway-dependent plasticity forms. These results are interesting and a direct comparison of IEGs*
110 *revealed important differences which suggest that different IEGs should be interpreted more specifically*
111 *with respect to the different pathways and associated forms of plasticity through which cortical neurons*
112 *can be activated. Still, a few points to be considered are listed below.*

113 *1. The first set of experiments was performed in normal mice with 24h dark adaptation, showing*
114 *moderate correlations between cell activity and IEG expression levels. Since the Authors argue that 60h*
115 *dark-adaptation is necessary to fully reveal the correlations, it is not clear why only 24h was used here?*
116 *As a minor aside, the Authors also argue that IEG expression better reflects peak than mean activity.*
117 *Then, why not the peak activities were shown in the main figures instead of relegating them to the*
118 *supplementary?*

119 **This may be a misunderstanding. The 24 hour dark adaption is one (of several) protocols that have**
120 **been used to induce IEG expression. As little as 3 hours of dark adaptation can induce c-Fos and EGR1**
121 **expression (Kaczmarek et al., 1999). The 60 hours dark adaptation is what a recent paper from the**
122 **Turrigiano lab reports is necessary to see increases in neural activity (very modest ones at that). Note,**
123 **the conclusion is that 24 hour dark adaption can induce increases in IEG expression levels, however,**
124 **these increases are not driven by increases in neuronal activity.**

125 **It is often assumed that IEG expression levels are a good proxy for mean activity. This has not been**
126 **validated directly of EGR1 or Arc, and only indirectly for c-Fos. The aim of figure 1 is to quantify the**
127 **relationship between IEG expression and average neuronal activity in vivo. Hence also the choice of**
128 **showing mean activity instead of max activity in the main figure.**

129 **This figure is somewhat orthogonal to the rest of the paper, but is undoubtedly of interest to people**
130 **using IEGs as markers for neuronal activity.**

131 *2. From the second set of experiments, the Authors reared mice in complete darkness until P40. It is not*
132 *entirely clear why the Authors moved away from the previous dark-adaptation paradigm to test 'first*
133 *visual experience' instead. Are the conclusions only valid with respect to the first visual/visuomotor*
134 *experience, or are there signs that they can be generalized to ongoing plasticity during natural visual*
135 *experience?*

136 **The aim of the paper was to address the question of whether visual and visuomotor experience**
137 **engage different plasticity mechanisms. This can only be done when animals have no previous visual**
138 **experience (i.e. are dark reared prior to the experiment). How the findings generalize to ongoing**
139 **plasticity during normal visual experience, we do not know.**

140 *3. Relatedly, how does the first visual exposure in life at P40 compare to naturally occurring first vision*
141 *(~P15?), with special concerns regarding visual critical period and plasticity rule changes during*
142 *development?*

143 **Dark rearing delays critical period plasticity in visual cortex. For experimental reasons we cannot**
144 **commence the experiments earlier (chronic head bar implants done too early result in severe skull**
145 **deformations). And the only way to experimentally record neuronal activity chronically around first**
146 **visual and visuomotor experience separately is to dark rear animals until they can be head bar**
147 **implanted. Using transgenic mice instead of AAVs one might be able to do the first experiment at**
148 **around P30, but that would still require dark rearing until that age. We know that a very similar**
149 **protocol to the one we use here, results in normal visuomotor integration in visual cortex (Attinger et**
150 **al., 2017; Widmer et al., 2022). Moreover, most of our conclusions in Figures 2-4 are based on**
151 **comparison between mice that all underwent the same dark rearing protocol.**

152 **We have added a point to the discussion related to this.**

153 *4. Xie et al. (2014) proposed that IEGs may be associated with pathway specific inputs. A more in-depth*
154 *discussion of this relatively closely related paper, as well as a clear demonstration of the advance in light*
155 *of those results upfront would be helpful.*

156 **We are not sure this is directly related, Xie et al (2014) report layer specific differences of EGR1**
157 **expression levels, not differences between functionally identified neurons, or correlations between**
158 **functional responses and IEG expression levels. Nevertheless, we now discuss the results of Xie et al**
159 **(2014) paper in the introduction and the discussion.**

160 *5. While not strictly necessary, showing that suppressing different IEGs leads to a differential*
161 *circuit/behavioral impact could substantially strengthen the conclusions. Can the Authors comment on*
162 *the possibility of such experiments?*

163 **These experiments are probably feasible in principle, using something like AAV mediated RNAi.**
164 **However, I would be very reluctant to try this, as IEGs likely have essential functions in cell**
165 **metabolism that go far beyond biasing input to either top-down or bottom-up. Either way, these**
166 **experiments would go far beyond the current study.**

167 *Minor: Line 77, ‘promotor’ should read ‘promoter’.*

168 **Corrected - thanks.**

169 **References**

170 Attinger, A., Wang, B., and Keller, G.B. (2017). Visuomotor Coupling Shapes the Functional Development
171 of Mouse Visual Cortex. Cell *169*, 1291-1302.e14.

172 Widmer, F.C., O'Toole, S.M., and Keller, G.B. (2022). NMDA receptors in visual cortex are necessary for
173 normal visuomotor integration and skill learning. Elife *11*, e71476.

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