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## Commentary: Fc Gamma Receptors are Expressed in the Developing Rat Brain and Activate Downstream Signaling Molecules upon Cross-Linking with Immune Complex

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During the past 20 years, a number of studies have reported IgG antibodies against viral or self-antigens in the developing human brain. Although the mechanisms by which they gain access to the developing brain are not yet clear, a subset of these antibodies has been linked to increased risk for neurodevelopmental disorders<sup>1–11</sup>. At first glance, such effects are expected to be mediated by IgG binding to its cognate Fc $\gamma$  receptors (Fc $\gamma$ R) on resident immune cells (such as microglia) in the brain and subsequent activation of local innate immune responses. However, considering that antibodies linked to neurodevelopmental disorders have subsequently been shown to recognize intracellular antigens expressed in neurons and astrocytes<sup>7</sup>, it is tempting to hypothesize that these autoantibodies could derail normal neurodevelopment by binding to their target antigens expressed on non-immune cells in the brain. Neuronal uptake of IgG antibodies against intracellular neuronal antigens has previously been shown and, in some cases, is thought to be mediated via clathrin-dependent endocytosis of IgG bound to Fc $\gamma$ R in these neurons<sup>12, 13</sup>. In combination with recent studies showing functional expression of Fc $\gamma$ RI in adult rat dorsal root ganglion neurons<sup>14–16</sup>, these findings prompted us to conduct the first comprehensive investigation of Fc $\gamma$ R expression and signaling on neurons and astrocytes in the developing rat brain. In our study, we documented sex-independent *in vivo* expression.

In our study we documented sex-independent *in vivo* expression of Fc $\gamma$ RIa, Fc $\gamma$ RIIa, Fc $\gamma$ RIIb, Fc $\gamma$ RIIIa, and Fc $\gamma$ RIIIb mRNA in the neonatal (postnatal days 1 and 7) rat cortex, hippocampus, and cerebellum at levels comparable to those observed in the spleen. Based on these findings, we investigated the functional expression of these Fc $\gamma$ R *in vitro* using primary co-cultures of neurons and astrocytes from neonatal rat pups. In both hippocampal and cortical cultures, we confirmed protein expression of Fc $\gamma$ RIa, Fc $\gamma$ RIIb, and Fc $\gamma$ RIIIa but not Fc $\gamma$ RIIIb in both cell types and showed that, similar to immune cells, expression of these receptors at the mRNA level is regulated by IFN $\gamma$ . Antibody-antigen IgG immune complexes (IgG-IC) triggered increased [Ca<sup>2+</sup>]<sub>i</sub>, a small but statistically significant increase

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in Erk phosphorylation, and Fc $\gamma$ R endocytosis in these cells, consistent with canonical Fc $\gamma$ R signaling in immune cells<sup>17–21</sup>. Calcium responses were concentration-dependent but showed high variability, consistent with [Ca<sup>2+</sup>]<sub>i</sub> transient variability in immune cells depending on cell type, activation state and ratio of excitatory to inhibitory Fc $\gamma$ R on the cell surface<sup>22, 23</sup>. The variability in [Ca<sup>2+</sup>]<sub>i</sub> responses can be further explained by our findings of subsets of neurons and astrocytes expressing either the excitatory Fc $\gamma$ RI, or the inhibitory Fc $\gamma$ RIIb or both. Although we were not able to detect intracellular IgG in neurons or astrocytes that endocytosed Fc $\gamma$ R following crosslinking with IgG-IC, inhibition of Fc $\gamma$ R endocytosis by pre-incubation with anti-Fc $\gamma$ RIa or anti-Fc $\gamma$ RIIb antibodies further supports that Fc $\gamma$ R signaling in immature neurons and astrocytes is triggered in the presence of immune complexes. Collectively, our findings of IFN $\gamma$ -regulated functional Fc $\gamma$ R expression by developing neurons and astrocytes underscore the need for further investigation into the role of Fc $\gamma$  receptors during normal and aberrant neurodevelopment.

The strength of our experimental approach is the functional characterization of neuronal/astrocytic Fc $\gamma$ R using IgG-IC to crosslink Fc $\gamma$ R on the cell surface and activate downstream signaling. Although IgG glycosylation is an important regulator of IgG-mediated immune signaling, monosaccharide IgG antibodies can also mediate effector functions in immune cells via Fc $\gamma$ R, but *in vitro* these functions can be predicted only when immune complexes are used<sup>24</sup>. In addition, with the exception of Fc $\gamma$ RI, which can be activated by monomeric IgG, all other IgG subclasses are activated in the presence of immune complexes but not by monomeric IgG (with the exception of FcRn, which functions to protect monomeric IgG from degradation)<sup>18, 25</sup>. To promote activation of all Fc $\gamma$ R isoforms in our study, and considering that we had no information regarding the glycosylation state of the IgG used to generate the immune complexes, we pre-incubated mouse IgG with rat anti-mouse IgG to generate IgG-IC across all our functional studies.

Despite this strength, there are potential gaps in our findings. Although the downstream effects we observed in our study (increased [Ca<sup>2+</sup>]<sub>i</sub>, Erk phosphorylation and Fc $\gamma$ R endocytosis) were triggered in the presence of IgG-IC, the IgG-IC concentration used to observe endocytosis was above physiologically relevant levels documented in rat cerebrospinal fluid (100  $\mu$ g/ml; although in humans such levels would be considered low)<sup>26–28</sup>, while similarly high concentrations of monomeric IgG also triggered [Ca<sup>2+</sup>]<sub>i</sub> and Erk phosphorylation but not Fc $\gamma$ R endocytosis. At the same time, the exact composition of the IgG-IC (ratio between rat IgG subclasses, IgG glycosylation state, and size of the immune complex) was not characterized and could have varied between individual preparations, thus affecting Fc $\gamma$ R crosslinking, activation and downstream signaling<sup>24, 26, 29</sup>. Another consideration is that the Fc portion of IgG can also be recognized by non-classical Fc-binding receptors on the cell surface. Such receptors have been described in immune cells (FcR-like 5 (FcRL5), Dectin-2, DC-Sign, MMR and MBL2)<sup>30</sup>, but the expression of such molecules in neurons/astrocytes in the developing brain has not yet been investigated. In summary, the specificity of Fc $\gamma$ R involvement in the IgG-IC-induced cellular signaling should be confirmed by knocking down Fc $\gamma$ R expression in these cells; this would also be useful for further assessing the specificity of the commercially available antibodies used to probe Fc $\gamma$ R protein expression in these cells. Studies of IgG-IC-mediated activation of additional signaling molecules downstream of Fc $\gamma$ R should also be conducted to identify

alignment with or potential deviations from canonical immune cell Fc $\gamma$ R signaling, which would further confirm a functional role of Fc $\gamma$ R in developing neurons/astrocytes. As an example, activation of neuronal Fc $\gamma$ RI in rat DRG neurons (which express Fc $\gamma$ RIa but not Fc $\gamma$ RIIb) results in increased [Ca<sup>2+</sup>]<sub>i</sub>, by triggering the non-selective cation channel TRPC3 through the Syk-PLC-IP<sub>3</sub> pathway<sup>15, 16</sup>. Although we probed for Syk phosphorylation in rat hippocampal and cortical neurons, we were not able to demonstrate Syk expression in these cells (unpublished data). The possibility that manual application of compounds to cultured neurons may trigger mechanosensitive receptor-mediated [Ca<sup>2+</sup>]<sub>i</sub> increase<sup>31</sup>, thus partially contributing to the observed effects, cannot be excluded. In addition, considering that hippocampal and cortical neurons express activating and inhibitory Fc $\gamma$ R on their surface, isoform-specific Fc $\gamma$ R knockdown and careful probing of downstream effectors would be useful in dissecting the relative contribution of each isoform on the downstream pathways activated<sup>32–34</sup>.

But what if the true role of neuronal/astrocytic Fc $\gamma$ R lies in bidirectional signaling with resident microglia and other immune cells in the brain? Expression of classical immune molecules such as MHC Class I proteins and complement receptors has been demonstrated to regulate microglia-mediated synaptic pruning during neurodevelopment<sup>35–37</sup>. Generating conditional neuronal Fc $\gamma$ R knockout models would be instrumental in assessing the existence of analogous interactions between neuronal Fc $\gamma$ R and microglia, and in understanding the role of neuronal Fc $\gamma$ R *in vivo*.

Going back to the original question of whether autoantibodies against intracellular brain antigens could derail normal neurodevelopment by engaging their intracellular targets via Fc $\gamma$ R-mediated uptake, there are additional questions that need to be addressed. Are these autoantibodies taken up by developing neurons in a non-stochastic manner? Are these autoantibodies already complexed with their target antigens when internalized? If not, is there another mechanism for their uptake into neurons which does not involve Fc $\gamma$ R engagement? Are these autoantibodies function-blocking? Alternatively, considering that non-specific monomeric IgG may be neuroprotective<sup>26</sup>, could this be the case also for maternal autoantibodies with specific brain antigen targets? Or are these autoantibodies simply diagnostic? Would neuronal subtypes with prevalent Fc $\gamma$ RI expression be more susceptible to uptake and effects of these antibodies? How would endocytosed IgG escape lysosomal degradation in neurons? Further studies addressing these questions are needed in order to assess a potential role for Fc $\gamma$ R in developing neurons and astrocytes in the reported association between maternal antibodies targeting developing brain antigens and increased risk for neurodevelopmental disorders.

In summary, hippocampal and cortical neurons express Fc $\gamma$  receptors and IgG immune complexes seem to crosslink these receptors, resulting in activation of downstream signaling. Although the functional readouts of IgG-IC priming of these cells are part of the immune cell signaling cascade normally activated by Fc $\gamma$ R crosslinking, further studies are needed to further elucidate the neurodevelopmental consequences of Fc $\gamma$ R expression in developing neurons and astrocytes and probe their potential activation by maternal autoantibodies against brain antigens.

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