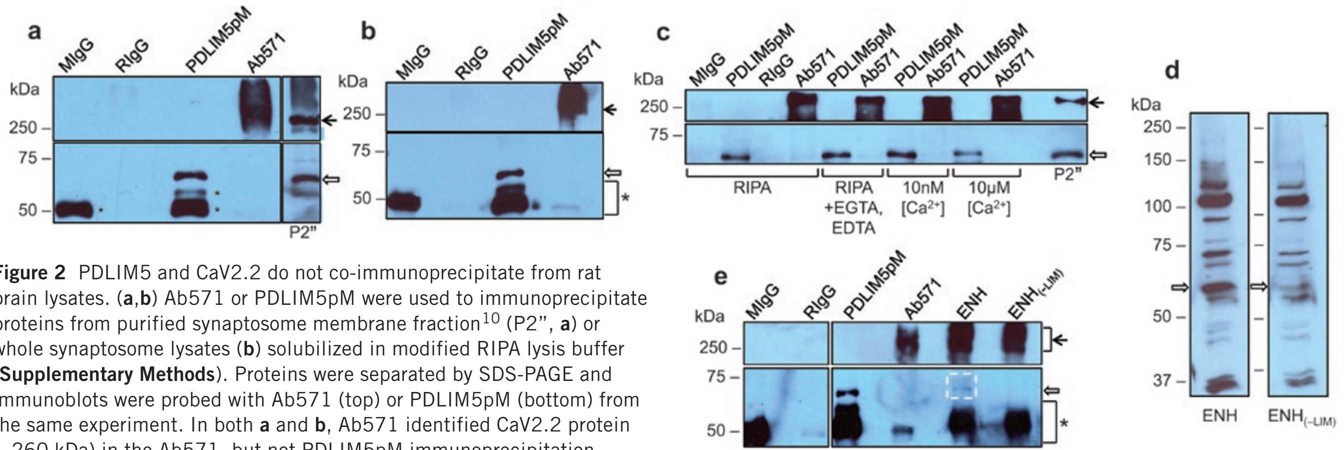

Figure 2 PDLIM5 and CaV2.2 do not co-immunoprecipitate from rat brain lysates. (**a**,**b**) Ab571 or PDLIM5pM were used to immunoprecipitate proteins from purified synaptosome membrane fraction[10] (P2", **a**) or whole synaptosome lysates (**b**) solubilized in modified RIPA lysis buffer (**Supplementary Methods**). Proteins were separated by SDS-PAGE and immunoblots were probed with Ab571 (top) or PDLIM5pM (bottom) from the same experiment. In both **a** and **b**, Ab571 identified CaV2.2 protein (~260 kDa) in the Ab571, but not PDLIM5pM immunoprecipitation, lanes and vice versa. A western blot of the P2" fraction identifying CaV2.2 and PDLIM5 protein bands is shown in the right lane of **a** (see also **Supplementary Fig. 2**). MIgG, mouse IgG; RIgG, rabbit IgG. (**c**) The P2" fraction was solubilized in RIPA lysis buffer with or without 3 mM EDTA and 5 mM EGTA and with the [Ca^{2+}] set to 10 nM or 10 μM. PDLIM5 failed to co-immunoprecipitate with CaV2.2 at all of the Ca^{2+} conditions tested. (**d**) Anti-ENH or anti-$ENH_{(-LIM)}$ were used to probe rat brain P2 western blots. (**e**) Ab571, PDLIM5pM, anti-ENH or anti-$ENH_{(-LIM)}$ were used for immunoprecipitation of proteins from adult rat brain crude membrane fraction and immunoblots were probed with Ab571 (top) or PDLIM5pM (bottom). PDLIM5 was seen in the PDLIM5pM and anti-ENH IP lanes, but not in the anti-$ENH_{(-LIM)}$ immunoprecipitation lane. CaV2.2 was detected in the Ab571 and anti-ENH lanes, as expected, and also in the anti-$ENH_{(-LIM)}$ immunoprecipitation lane. The white dashed box indicates the location of the PDLIM5 band. The closed arrow indicates CaV2.2 and the open arrow indicates PDLIM5. * indicates the antibody protein bands. Blots grouped together are all from the same experiment. Anti-ENH and anti-$ENH_{(-LIM)}$ are referred to as ENH and $ENH_{(-LIM)}$, respectively.

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CaV2.2, (when probed with PDLIM5pM; **Fig. 2e** and **Supplementary Fig. 6**). However, we had reservations as to whether this truly reflected co-immunoprecipitation with PDLIM5 because the reverse, Ab571 immunoprecipitation, probing with ENH did not recover PDLIM5 (**Supplementary Fig. 6**). This reservation was confirmed by repeating the immunoprecipitation experiment after depleting antibody to PDLIM5 clones from anti-ENH using LIM1-3 immobilized on beads (termed anti-$ENH_{(-LIM)}$; **Fig. 2d**). Depletion was confirmed by a very weak band corresponding to the molecular weight of PDLIM5 in western blots (**Fig. 2d**) and its absence when used to probe immunoprecipitated proteins (**Fig. 2e** and **Supplementary Fig. 6**). However, despite the gross reduction in the antibody to PDLIM5 fraction, $ENH_{(-LIM)}$ still immunoprecipitated CaV2.2 (**Fig. 2e**). There were two simple explanations for this finding. Either anti-ENH contains antibody clones that bind directly to CaV2.2 or it captures the channel as a binding partner of a target protein other than PDLIM5. We favor the latter interpretation, as neither anti-ENH nor anti-$ENH_{(-LIM)}$ identified a band with a molecular weight that corresponds to CaV2.2 in western blots of brain lysates (**Fig. 2d**).

Thus, although we confirmed that PDLIM5 is present in presynaptic terminals, our electrophysiology, immunocytochemistry and biochemistry results argue against the idea that this protein serves as an adaptor to link PKCε to CaV2.2. It should be noted that an important tool in our study, the highly specific and avid antibody to PDLIM5, PDLIM5pM, was not available at the time of the original study[2] and the capture of CaV2.2 using their antibody to ENH would have been compelling evidence in support of such a complex. Hence, the CaV2.2-to-PKCε adaptor role for PDLIM5 was not unreasonable at that time. However, the results presented here do not support this hypothesis and instead indicate a re-examination of the function of PDLIM5 in the brain and also specifically at the presynaptic terminal, a quest that becomes more important by the reported association of this protein with serious psychiatric disorders.

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1. Kuroda, S. *et al. J. Biol. Chem.* **271**, 31029–31032 (1996).
2. Maeno-Hikichi, Y. *et al. Nat. Neurosci.* **6**, 468–475 (2003).
3. Chen, Y., Lai, M., Maeno-Hikichi, Y. & Zhang, J.F. *Cell. Signal.* **18**, 215–224 (2006).
4. Iga, J. *et al. Neurosci. Lett.* **400**, 203–207 (2006).
5. Li, C. et al. Int. J. Neuropsychopharmacol. **11**, 27–34 (2008).
6. Horiuchi, Y. *et al. Biol. Psychiatry* **59**, 434–439 (2006).
7. Khanna, R., Li, Q., Bewersdorf, J. & Stanley, E.F. *Eur. J. Neurosci.* **26**, 547–559 (2007).
8. Li, Q. *et al. J. Neurosci.* **24**, 4070–4081 (2004).
9. Khanna, R., Zougman, A. & Stanley, E.F. *J. Biochem. Mol. Biol.* **40**, 302–314 (2007).
10. Khanna, R., Li, Q., Sun, L., Collins, T.J. & Stanley, E.F. *Neuroscience* **140**, 1201–1208 (2006).

Maeno-Hikichi *et al.* reply: Our original conclusion that the PKC binding protein, enigma homolog (ENH), interacts specifically with both PKCe and N-type Ca^{2+} channels, forming a PKCε-ENH–Ca^{2+} channel macromolecular complex was based on a polyclonal antibody that used the last 18 amino acids of ENH as an epitope. We do not have any more stock of this original antibody. The Abnova antibody used by Stanley and colleagues was raised against the entire ENH protein (a total of 597 amino acids) and this may explain some of the differences between our results. However, the antibody that we supplied to Stanley and colleagues was an antibody that was similar to the one that we used in our original study and was also raised against the last 18 amino acids of ENH. We are not sure why Stanley and colleagues found this antibody to be nonspecific. In addition, it is not clear to us why inclusion of a fusion protein containing ENH binding domains in their recording pipette did not facilitate modulation of N-type Ca^{2+} channel activity by PKC in the recorded rat DRG neurons.

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