Detonations of small spherical explosive charges disrupt synaptic integrity and NMDA receptor-cofilin signaling in the absence of neurodegeneration in hippocampal slices.

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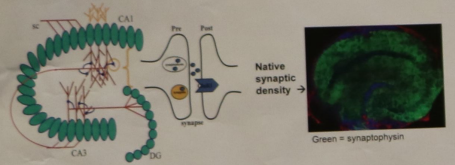
Abstract

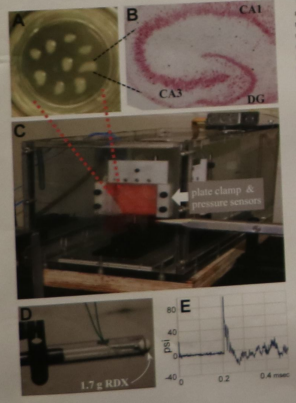
Primary explosive blast waves cause common types of military-related traumatic injuries. A novel procedure was developed that generates distinct blast waves from 1,3,5-Trinitroperhydro-1,3,5-triazine (RDX), explosive (Zander et al. 2015 *J Neurosci Res* 93:1353; Smith et al. 2016 *Exp Neurol* 286:107). To study direct effects of detonated RDX spherical charges on brain tissue, rat hippocampal slice cultures were sealed in serum-free medium, lowered into a water-filled tank, and RDX assemblies detonated outside the tank. Compared to mock-treated slice cultures, the RDX blasts caused a dose-dependent reduction in pre- and postsynaptic markers. GluR1 was reduced by 25-40% after two consecutive blasts 4 min apart, and further reduced by 60-70% after three RDX blasts. The degree of synaptic marker loss correlated with increased levels of HDAC2, a histone deacetylase implicated in stress-induced reductions in glutamatergic transmission and recognition memory (Wei et al. 2016 *J Neurosci* 36:2119). The blast-induced loss of GluR1, NCAM180, and synaptophysin was rapid, with 40-50% declines evident 24 h after multiple detonations, followed by further decline at 45 h post-blast, and no indication of recovery at 72 h. Interestingly, while protein accumulation events (e.g. tau, TDP-43) are often common features of TBIs (Blennow et al. 2012:*Neuron*; Goldstein et al. 2012:*Sci Transl Med*; Smith et al. 2013: *Nature Rev Neurol*; Zhang et al. 2015:*J Cereb Blood Flow Metab*), the synaptic decline temporal profiles induced by the small 1.7-g RDX explosives aligned much closer to synaptic decline profiles from excitotoxic hippocampal slices than to the declines in protein accumulation stress studies. However, the RDX blast-induced disturbances in synaptic integrity were observed in the absence of calpain-mediated cytoskeletal damage and neuronal death, events tightly associated with synaptic marker loss after hippocampal excitotoxicity (Bahr et al. 2002, *Exp Neurol* 174:37). Also, Fluoro-Jade B staining found no indication of degenerating neurons in slices exposed to three RDX blasts, suggesting that certain levels of military explosives produce a unique type of pathology comprised of altered synaptic integrity before cellular deterioration. The results suggest that, in addition to serious brain damage from large explosions, primary blast impacts can cause synaptic compromise without producing overt neurodegeneration, perhaps explaining the cognitive and behavioral changes in blast-induced TBI sufferers with no detectable neuropathology. The monitoring of individuals' cumulative blast exposures is a critical issue in light of this study. Also, this work will help understand how military blasts might increase the risk of Alzheimer's disease.

1. Native synaptic density in hippocampal slice culture model



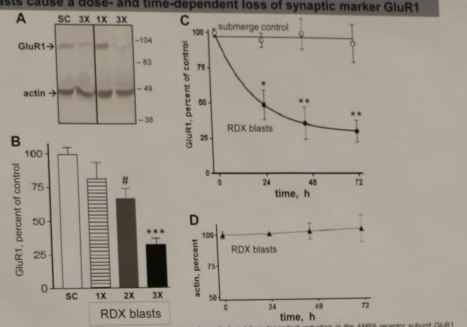
Synaptic circuits of the hippocampal network show the different subregions: DG, CA3, and CA1. Additionally, the most widely studied synaptic proteins synaptophysin, GluR1 and synapsin II are illustrated (left). Native synaptic density can be seen in a synaptophysin stained hippocampal slice.

2. Experimental model to produce RDX blast shockwaves



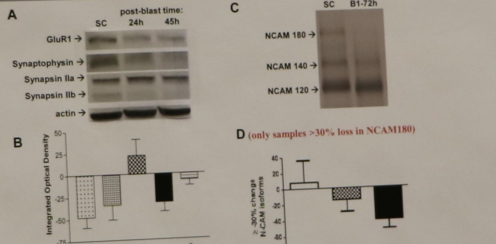
Hippocampal slice cultures were used to study the effects of realistic blast waves. Slices at culture day 4 are shown distributed on a culture insert membrane (A). The long-term slice cultures display features of the adult brain including intact hippocampal subfields visualized by H and E staining (B; culture day 28). Slice cultures in a six-well plate were sealed in SFM and clamped in position within a warmed, water-filled chamber (C). Three piezoelectric, high frequency pressure sensors are located directly above the clamped culture plate. A spherical assembly of RDX explosive (D) was detonated outside the chamber and the generated blast wave traveled through the wall of the blast chamber, into the water medium, and was measured as a pressure history profile (E). DG, dentate gyrus.

3. RDX blasts cause a dose- and time-dependent loss of synaptic marker GluR1



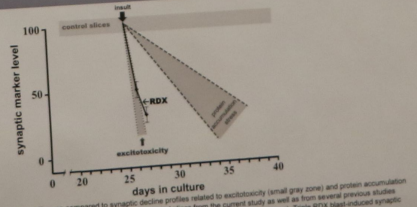
Military explosive blasts from 1.7-gram RDX assemblies cause a dose-dependent and time-dependent reduction in the AMPA receptor subunit GluR1. Hippocampal slice cultures exposed to 1–3 RDX blasts (1×–3×) were assessed together with mock submerge control slices (SC) by anti-GluR1 immunoblotting (A). Each blot was subsequently stained for actin for a protein load control, and positions for molecular weight standards are shown for 38–104 kDa. GluR1 immunoreactivities (means±SEM) are shown as percent of levels found in submerge control groups of slices (B) (ANOVA: $p<0.0001$; compared to submerge control slices: #$p=0.011$, ***$p<0.0001$). Other slice cultures were subjected to RDX detonations or mock treatment, then harvested to assess GluR1 levels across post-blast times (C) (two-way ANOVA: $p<0.0001$, comparison between control and RDX blast groups at each time point: *$p< 0.05$; **$p<0.01$). The blast samples were also assessed for actin levels (D).

4. Blast shockwaves cause distinct changes in synaptic proteins



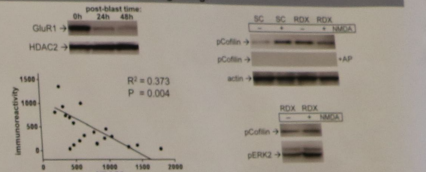
Multiple RDX blasts induce distinct changes in synaptic proteins. Hippocampal slice cultures were subjected to 3 RDX detonations then harvested over time in order to assess five synaptic markers and actin by immunoblot within a subset of blast and mock treatment samples. Within-blot immunoreactivity values were normalized to the mock treated control samples for each synaptic marker (means±SEM). SC, submerge control group. The distinct changes in 4 synaptic markers were assessed by ANOVA: $p=0.0196$. The increase in synapsin 2a was not significant (1-sample t test: $p=0.29$). NCAM isoforms are differentially altered by RDX detonation. Western blot analysis demonstrates a decrease in NCAM 180 and 140 in response to a blast insult (C) cumulative quantification (n=9) confirmed western blot results. ($p=0.1184$) In samples that demonstrated a greater than 30% reduction (n=6) in NCAM 180 (D), nonparametric ANOVA (Kruskal-Wallis test) indicates significant differences among the isoforms ($p=0.0027$). Dunn's Multiple Comparisons to NCAM180: $p<0.001$ for NCAM120; $p<0.05$ for NCAM140.

5. Blast-induced synaptic decline closely matches excitotoxic-related synaptic loss



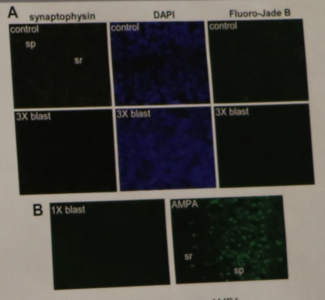
The blast-induced synaptic decline profile was compared to synaptic decline profiles related to excitotoxicity (small gray zone) and protein accumulation stress (large gray zone) summarized from previous hippocampal slice. Control slices from the current study as well as from several previous studies together showed that synaptic marker levels were stable across the period of 10–40 days in culture (hatched area). Triple RDX blast-induced synaptic marker loss is shown with the solid black line.

6. Blast-induced synaptic decline occurs with enhanced histone deacetylase levels and disrupted NMDA receptor-cofilin signaling.



RDX blast-induced loss of GluR1 immunoreactivity in brain tissue samples corresponds with increased levels of the HDAC2 protein. The immunoreactivity levels of the synaptic marker and HDAC2 across individual slice samples were plotted against each other and linear regression resulted in a significant correlation ($R2 = 0.373$; two-tailed, one-sample t-test on resulting slope: $p= 0.004$). (Left) At 24 h post-blast, slice cultures treated with 2 RDX blasts and control slices were infused with 100 µM NMDA for 5 min and harvested in buffer containing phosphatase inhibitors. The identical samples on two nitrocellulose strips were incubated with or without alkaline phosphatase (AP) for 2 h at 37 °C before phospho-cofilin immunostaining (Right, top). Each blot was subsequently stained for actin. Another set of double-blast slices were subjected to the NMDA treatment to assess pCofilin and pERK2 in the same slice samples (Right, bottom).

7. Synaptic decline occurs without evidence of cellular damage after blast exposure



Hippocampal slice cultures from a control mock treatment group and a triple-blast group were fixed 45 h after the detonation events and assessed for anti-synaptophysin immunostaining and DAPI counterstaining, and images from the same CA1 area are shown (view-field width: 180 µm). The same slices were also subjected to Fluoro-Jade B staining and a larger area imaged due to no cellular staining evident (A; view-field width: 400 µm). A positive control for the Fluoro-Jade stain consisted of slice cultures treated with 100 µM AMPA for 15min and the tissue fixed 48 h post-insult for CA1 imaging (B). In the same area of a slice subjected to a single RDX blast, Fluoro-Jade B did not stain any degenerating pyramidal neurons (view-field width: 450 µm). sp, stratum pyramidale; sr, stratum radiatum. Spectrin breakdown product (BDP) was assessed by immunoblot in a positive control sample of calcain-mediated BDP (pos) and in hippocampal slices from the submerge control group (SC), a triple-blast group (3X), untreated control group (con), and AMPA-insult group (C).

Conclusion

- The results of this study suggest hippocampal slices that received two consecutive RDX blasts 4 min apart exhibited reductions in GluR1, and it was further reduced after three consecutive blasts.
- The presynaptic marker synaptophysin was as susceptible as the postsynaptic protein GluR1, 24 h after three detonations and further reduced 45 h post-blast. Interestingly, synapsin 2b exhibited a marked decline as well, whereas synapsin 2a was unaffected. Also, synaptic decline from the RDX blasts was found to correspond more closely with synaptic deterioration from excitotoxicity events than abnormal protein accumulation.
- RDX detonations disrupt the NMDA-mediated pCofilin pathway that is linked to long-term potentiation and memory encoding.
- Fluoro-Jade staining found no indication of degenerating neurons in slice cultures exposed to three RDX blasts, suggesting that low-level explosives can produce synaptic alterations in the absence of cellular degeneration.
- Together, these results indicate that detonated RDX explosives cause distinct losses of synaptic proteins before cell death, perhaps explaining the cognitive deficits in those blast-induced TBIs with no detectable neuropathology.

Acknowledgements & References

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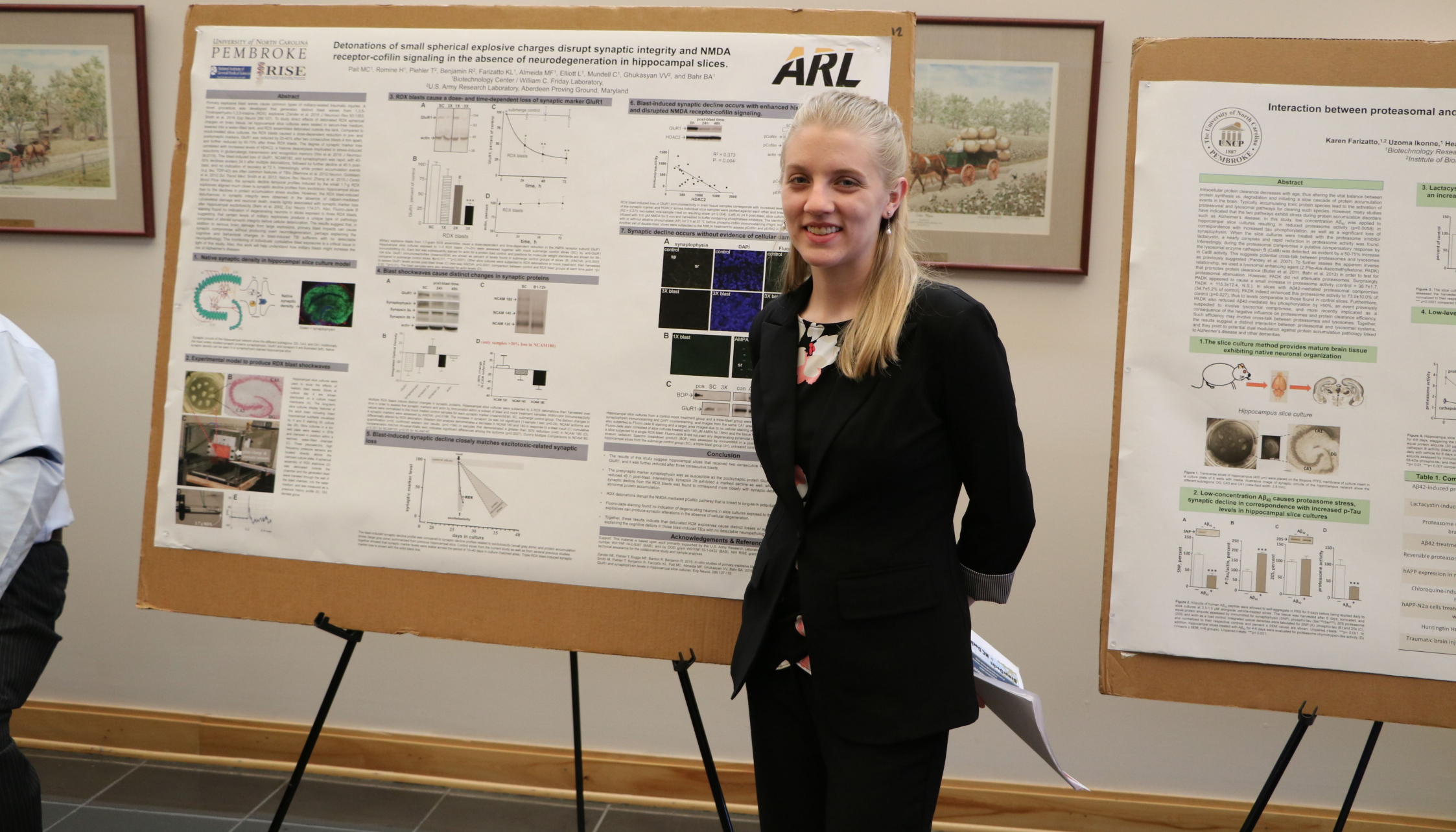
Smith M, Piehler T, Benjamin R, Farizatto KL, Pait MC, Almeida MF, Ghukasyan VV, Bahr BA. 2016. Blast waves from detonated military explosive reduce GluR1 and synaptophysin levels in hippocampal slice cultures. Exp Neurol. 286:107-115.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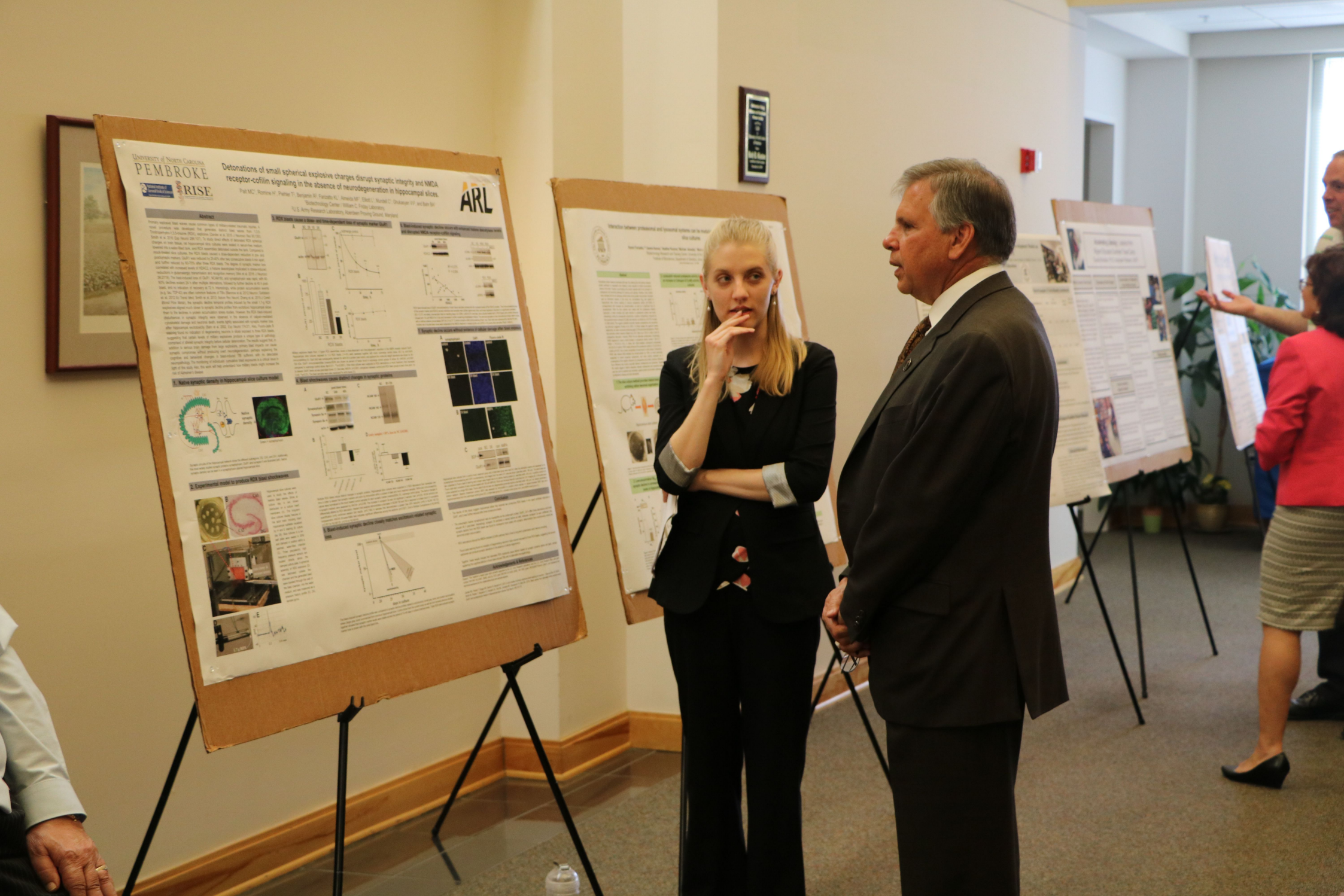
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