Scientific context on the 2009 paper (Nikolaev et al. Nature 2009) **and subsequent work**
February 15, 2023

The following provides a brief summary of the 2009 paper and subsequent work, followed by a detailed discussion with references that support the statements in the summary.

Summary:

- The 2009 paper reported involvement of DR6, APP and Caspase-6 in developmental axon degeneration, and proposed that DR6 and APP interact biochemically and in the same pathway. These findings, all of which were novel, have been fully validated. They have also been found, for DR6 and APP, to extend beyond development to synapse regulation and axonal pruning in normal adult brain.
- The 2009 paper reported that Caspase-3 inhibitors do not block degeneration, replicating the independent work of Raff and colleagues. However, our later analysis using knock-out mice showed that, contrary to their and our belief, Caspase-3 is in fact involved, and provided an explanation for the difference between inhibitors and knockout.
- The 2009 paper also proposed a specific model for how APP and DR6 interact that involves cleavage of APP by β-secretase and interaction of an amino-terminal fragment of APP (N-APP) with DR6. Key experiments in support were that β-secretase inhibitors and anti-N-APP antibodies blocked degeneration, and an independent replication of those findings was published the following year by Milbrandt and colleagues. However, our further extensive analysis using knock-out mice and additional biochemistry revealed that their and our conclusions had been affected by off-target actions of several of these reagents, and led us to propose a revised model for the interaction, which has since received support from structural studies.
- The 2009 paper also speculated whether the DR6-APP mechanism might contribute to axon degeneration in neurodegenerative disease; this possibility has received some support for Amyotrophic Lateral Sclerosis (ALS).

In short, central tenets of the 2009 paper – the involvement of DR6, APP and Caspase-6 in developmental degeneration, and the fact that DR6 and APP interact biochemically and in the same pathway – have been validated and extended to normal adult brain and neurodegenerative disease. We were led to revise our initial models for activation of Caspase-6 (which actually involves Caspase-3) and for the APP-DR6 interaction, as our new data led to new understanding.

A detailed discussion follows on the next page.

Detailed discussion:

1. **The 2009 paper reported the following findings that have been fully validated** (e.g., Simon et al. J. Neuroscience 2012; Olsen et al. J. Neuroscience 2014, among others):

- The novel discovery that a Death receptor, DR6, regulates developmental axon degeneration.
- The novel discovery that the Amyloid Precursor Protein, APP, regulates developmental axon degeneration.
- The novel discovery that DR6 and APP interact biochemically and in the same pathway to regulate developmental axon degeneration.
- The novel discovery that a Caspase, Caspase-6, regulates developmental axon degeneration.

2. **Two aspects of the 2009 paper were revised by subsequent experiments**:

- **Regarding Caspase activation**: based on use of acute inhibitors, the 2009 paper had concluded that Caspase-3, a well-known executioner, was not involved, thereby replicating findings by Martin Raff and colleagues (Finn et al. J. Neuroscience 2000), who had forcefully concluded that Caspase-3 is not involved in developmental axon degeneration. In our follow up study (Simon et al. J. Neuroscience 2012), however, we discovered that Caspase-6 is actually activated by Caspase-3, and, using knock-out mice, that the latter does in fact participate in degeneration. We also found the reason that Caspase-3's involvement had been missed by Raff and by us is because degeneration can be blocked only by full genetic knockout, not by the partial inhibition afforded by inhibitors or knockdown used in earlier studies. We further provided evidence that the need for full genetic knockout reflects the fact that Caspase-3 acts catalytically, an important aspect of the mechanism. As the paper states (see end of Introduction, page 17541): *"Unexpectedly, we found that Caspase-3 plays an obligate role in axon degeneration following NGF withdrawal in vitro. Our data suggest that only small amounts of Caspase-3 are necessary to fully process Caspase-6 and initiate degeneration, providing an explanation for the lack of detection or appreciable inhibition seen in other studies (Finn et al., 2000; Plachta et al., 2007; Nikolaev et al., 2009). Our studies also implicate Caspases-3 and -6 in developmental axon pruning in vivo."*

- **Regarding the DR6-APP interaction**: the 2009 paper proposed an initial model for how the two proteins interact, in which activation involves cleavage of APP by β-secretase followed by binding of an amino-terminal fragment of APP (N-APP) to DR6. This was based on multiple lines of evidence, including biochemical analysis and the fact that cleavage and degeneration were blocked by three chemically distinct inhibitors of β-secretase, as well as by antibodies to DR6 and N-APP. By the next year, Milbrandt and colleagues had replicated the degeneration block we saw using β-secretase inhibitors and anti-N-APP antibodies (Vohra et al. J. Neuroscience 2010; see Figure 1A and 1C),

supporting our model. Our follow up study (Olsen et al. J. Neuroscience 2014) also fully replicated the inhibitory effects of these reagents. However, the Olsen 2014 paper, which made extensive use of knock-out mice as well as further biochemical analysis, also unexpectedly showed that a number those inhibitors and other reagents had off-target effects that affected Milbrandt's and our conclusions. A very detailed analysis then led us to propose a revised model in which cleavage by β-secretase is not required for activity, and a more C-terminal portion of APP is key to the interaction. The paper explains in extensive detail how new data led to this revised model; a long excerpt from the paper is provided below that lays out some of those details that are particularly pertinent to the revision of the role of N-APP (see footnote 1). Our revised model for biochemical interaction of DR6 and APP was later supported by structural (crystallographic) data (Xu et al., Genes Dev. 2015).

3. **Involvement of the DR6-APP mechanism has been extended beyond development to the healthy adult brain** in studies showing that:

- DR6 regulates excitatory synaptic spine density in adult brain in a genetic pathway with APP (Kallop et al. J. Neuroscience 2014);
- DR6 and APP regulate not just developmental degeneration/pruning but also pruning in adult cerebral cortex during experience-dependent plasticity (Marik et al. J. Neuroscience 2013; Marik et al. PNAS 2016).

4. **The 2009 paper also speculated that the DR6-APP interaction might participate in axon degeneration during disease, a possibility that has garnered support**.

- In a first test led by our collaborators at Genentech, removing DR6 did not alter pathophysiology in two specific mouse models of Alzheimer's Disease (AD) (Kallop et al. J. Neuroscience 2014). However, since those models, like all mouse AD models, have limited predictive value – in particular, they do not involve actual axon degeneration – this result did not exclude a possible role for DR6 in some aspects of AD or other neurodegenerative diseases. As the paper states in its concluding two sentences: *"Our results, therefore, argue against a central role for DR6 in establishing APP and/or Aβ driven pathology in these mice. Whether it contributes to the axon degeneration observed in Alzheimer's disease remains to be determined."* That said, since it is important to have preclinical models to test therapeutic candidates, the failure to see protection in this model effectively made further progress on a therapeutic program targeting DR6 very difficult.
- Importantly, though, a later study by an independent group converged on the DR6-APP mechanism as a contributor to neurodegeneration in Amyotrophic Lateral Sclerosis (ALS, or Lou Gehrig's disease) (Mishra et al. Nature Communications 2020). Their results also supported the revised model for how DR6 and APP interact.

5. Finally, at every stage, these findings were discussed and debated in the scientific community.

- An example is coverage of our back-to-back 2014 papers with our collaborators (Olsen et al. J. Neuroscience 2014; Kallop et al. J. Neuroscience 2014).

Footnote 1: A detailed discussion of the key observations that explain the shift from the initial model to the revised model for DR6-APP interactions is provided in the Results and Discussion sections of Olsen et al. J. Neuroscience 2012. Here, select portions that are relevant to the role of N-APP are reproduced, but the entire paper should be examined to get the full picture.

From page 6444:

"…Given the evidence that β-secretase activity is not required for degeneration, we revisited the biochemical (binding) and functional (prodegenerative) effects of N-APP. The prior study (Nikolaev et al., 2009) had used N-APP from two sources, commercial (Thermo Fisher) and in-house (Genentech), which gave consistent results. However, both preparations were only partially purified and biochemical analyses revealed them to contain contaminants and aggregated material (data not shown). To guard against nonspecific effects, we set out to obtain purer and nonaggregated N-APP. As purification proceeded, we unexpectedly found that the prodegenerative effects of N-APP were lost, as was binding to the DR6 ectodomain (fused to alkaline phosphatase: DR6-AP) observed by ELISA (Fig. 7E). One possibility is that the binding and functional effects seen with earlier material were caused by aggregates that may have accumulated during partial purification; an alternative is that a contaminant in the partially purified material contributed to activity and/or binding."

(…) Because we consistently observe robust and reproducible binding of DR6-AP to COS-7 cells expressing full-length APP (Nikolaev et al., 2009; Fig. 7), the lack of binding of more purified N-APP to DR6-AP suggested that the N-terminal portion of APP might not be the major mediator of the interaction. We therefore performed a more extensive structure–function analysis. (…)"

and further on page 6446:

"In fact, further biochemical and more detailed structure–function analysis indicates that the high-affinity biochemical interaction of the full APP and DR6 ectodomains consistently observed in cell-based binding assays actually requires a portion of the APP ectodomain that is more C-terminal than previous appreciated (C-APP), residing in its so-called E2 domain; however, more N-terminal sequences could also potentially contribute, because the affinity of binding of C-APP was lower than that of the full APP ectodomain (Fig. 7)."