The novel NMDA receptor modulator NYX-2925 enhances dendritic spine-autonomous structural and functional plasticity *in vitro*

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SUMMARY

On principal excitatory neurons, structural plasticity involves both alterations in the size of existing spines (enlargement or shrinkage in response to LTP/LTD, respectively) as well as the formation and elimination of spines (spine stability/turnover or dynamics). These structural changes manifest in response to functionally relevant stimuli and are therefore believed to be the structural correlate of learning and memory. Whereas these adaptive responses are integral components of Hebbian learning, they are also altered in neurological conditions such as severe/prolonged stress and related PTSD, brain injury (TBI and stroke), epilepsies, and genetic abnormalities (Fragile X, Rett, etc.). Mechanisms governing structural plasticity appear to overlap with functional plasticity (e.g. AMPA receptor insertion), but have yet to be fully elucidated. We have developed a library of novel NMDA receptor modulators that enhance functional plasticity in behavioral models of learning and memory and electrophysiological models such as potentiation of CA1 field potentials (classical LTP). However, whether these compounds modulate structural plasticity acutely or in models of neurological disease is unknown. To assess the role of our lead compound NYX-2925 on structural plasticity in excitatory spines, we have employed a high-frequency glutamate uncaging (HFU) protocol in cultured rat neurons that is known to induce structural LTP (sLTP) in isolated spines. We find that NYX-2925 robustly enhances NMDA receptor-dependent increases in spine volume. The enhancement of sLTP is evident both in the magnitude of change in spine volume and the number of spines that respond to sLTP induction. Furthermore, to determine if NYX-2925 enhances plasticity-related AMPA receptor insertion (a direct measure of functional plasticity), we

METHODS

Primary neuronal cultures: Embryonic cortical or hippocampal neurons were grown on poly-d lysine coated 12mm coverslips for ICC (cLTP), 18mm coverslips for live-cell imaging (sLTP and cLTP), or 10mm plates for biochemistry experiments.

Chemical LTP (cLTP): Cultures were exposed to 200µM glycine in Mg^{++}- free HEPES-buffered ringer solution in the presence of TTX and bicuculline for 3-5min and allowed to recover for 20min. Control experiments were performed in the absence of glycine.

Structural LTP (sLTP): Primary neuronal cultures were transfected with dsRED2 (fill, volume measurements) and GCaMP6s (to detect Ca^{++} in responsive spines) and NMI-caged glutamate was uncaged with point stimulation ~0.2-0.5µM from spine head using minimal stimulation (1-2% laser power, 1-2msec, 405nm line). Prior to and 3min after a 1Hz, 30X glutamate uncaging, high-resolution z-stacks were acquired every 3min for 30min.

Immunocytochemistry (ICC): Primary neuronal cultures were fixed with 4%PFA/4% sucrose at 4˚C for 12min, blocked with 10% donkey serum and incubated with anti-N-terminal GluA1 @ 4˚C for 3h. Following extensive washes and permeabilization, anti- PSD95 was incubated for 3-12h @4˚C, followed by appropriate fluorescently conjugated secondary antibodies.

Biochemistry: Following cLTP induction, cultures were rinsed with ice-cold HBSS 2X and monolayers collected in 320mM sucrose. Homogenization was performed with a 26g syringe. Homogenates were centrifuged at 500g for 5min and post nuclear supernatants at 17kxg for 30min. P2 pellets were resuspended in PBS with 1% Triton-X100 and agitated for 30min at 4˚C. Prior to centrifugation at 32kxg for 20min, a sample was taken for total protein (I=input) and following centrifugation, soluble (S) and insoluble (P, pellet) fractions were normalized to initial volume for further analysis by Western blot.

Image analysis and Microscopy: Images for live-cell experiments and fixed cultures were acquired with a Nikon A1R equipped with a piezo stage and analyzed using Volocity 6.3.

Statistics: 1- or 2-way ANOVA (repeated measures where relevant) was performed with Bonferroni post hoc analysis. P<0.05 was considered statistically significant.

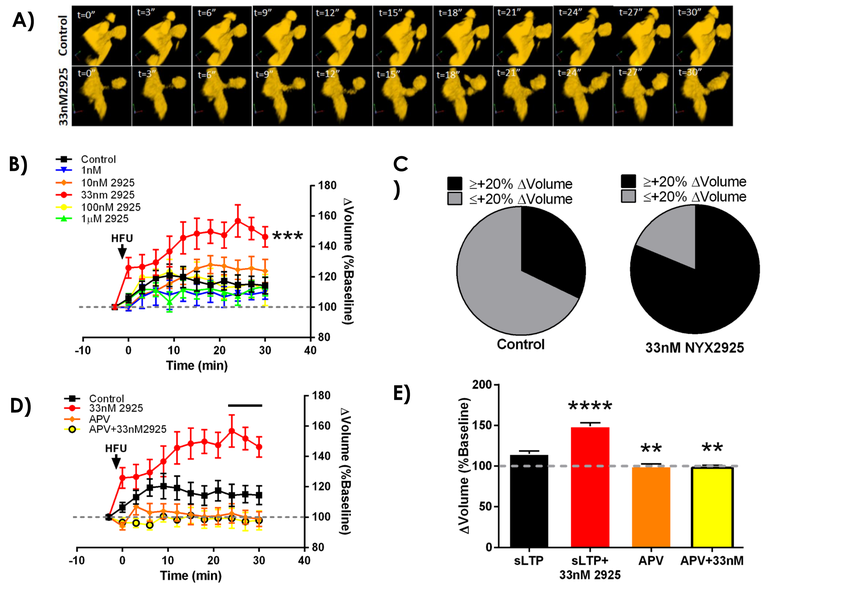
CONCLUSIONS

- NYX-2925 enhances structural and functional plasticity *in vitro*.
- NYX-2925-dependent plasticity requires engagement of NR2B *in vitro*.
- These findings inform the mechanism by which NYX-2925 may exert therapeutic effects and highlight the clinical potential of compounds that facilitate NMDA receptor-dependent structural plasticity.

FINANCIAL DISCLOSURES

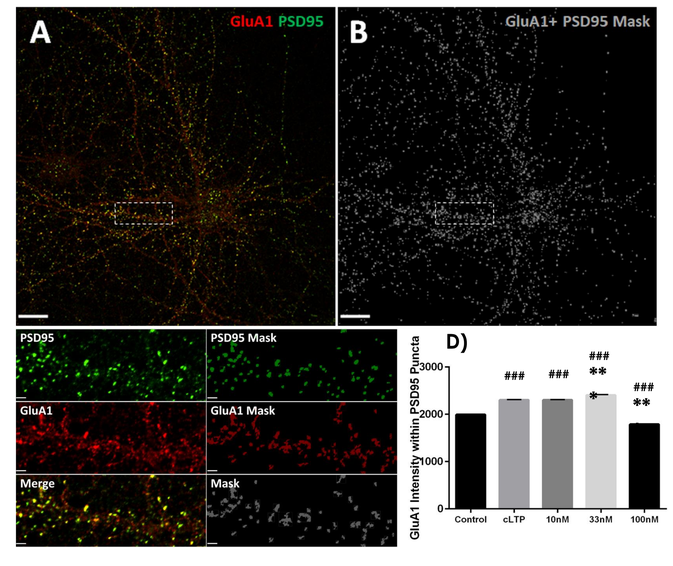
R.M. Mitchell, L.P. Cacheaux, M.S. Bowers, A.I. Shanker, R.A. Kroes, and J.R. Moskal are employees of Aptinyx, Inc., Evanston, IL.

NYX-2925 augments dendritic spine-autonomous structural plasticity



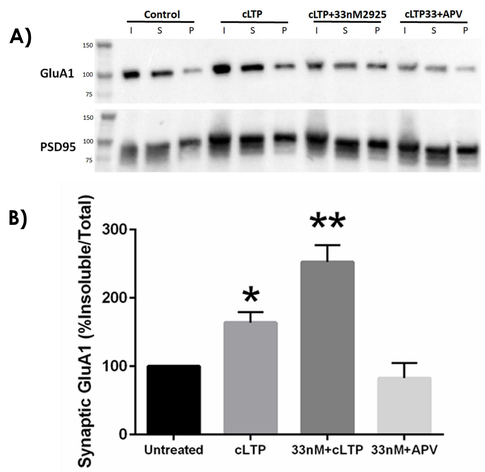
DIV 14-16 cortical cultures were co-transfected with dsRED to fill neurons and GCaMP6s to detect Ca^{2+} transients for 24-72h. NMI-caged glutamate was uncaged ~0.2-0.5µm from isolated spine heads 30x @ 1Hz (high-frequency uncaging, HFU) using minimal stimulation (1-2ms, 1-2% power) with a 405nm laser line. High-resolution z-stacks were acquired every 3min and spine volume was analyzed with Volocity 6.3. **A)** Representative time-lapse images of spine volume from control and 33nM NYX-2925- treated experimental groups. **B)** Dose-response volume data for sLTP in the presence of increasing concentrations of NYX-2925. Note the robust potentiation of spine volume in the presence of 33nM NYX-2925, p<0.001 vs. control. **C)** Pie charts showing the population of spines that increased >20% between control and 33nM sLTP groups. **D)** sLTP and potentiation of spine volume were blocked by the competitive NMDAR antagonist APV. **E)** Quantification of data in D for time points indicated by black bar.

NYX-2925 enhances functional plasticity in vitro



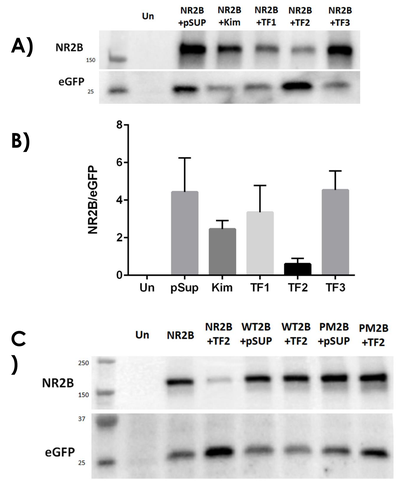
DIV14-18 cortical cultures were either mock-treated or exposed to cLTP (200µM glycine) in the presence of increasing concentrations of NYX2925. **A)** Representative immunocytochemical image of surface GluA1 (red) and PSD95 (green) staining. **B)** PSD95 and GluA1 positive surfaces were analyzed independently in Volocity 6.3 and GluA1 negative PSD95 surfaces were excluded. **C)** Enlarged segment of dendrite (rectangle) in A&B showing confocal images (left) and Volocity mask (right) used for quantification. **D)** Population data of dose-response for cLTP experiments in the presence of increasing concentrations of NYX-2925. Note the enhancement of cLTP-induced synaptic GluA1 with 33nM NYX-2925. Representative of three independent experiments, 10 neuronal fields/group, ### p<0.0001 vs. control, ***p<0.0001 vs. cLTP. Scale bar A&B= 30µm, C= 1µm.

NYX-2925 enhances cLTP-induced, biochemically defined, synaptic GluA1



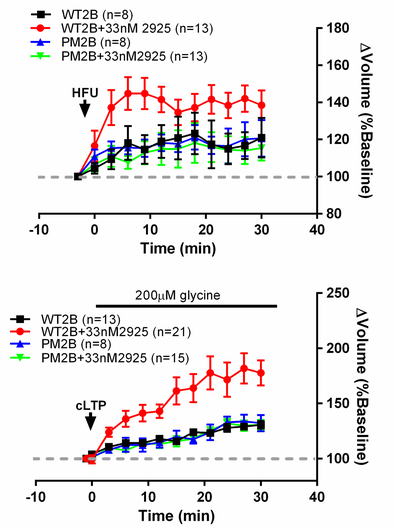
DIV14-18 cortical or hippocampal cultures were untreated or exposed to cLTP alone, in the presence of 33nM NYX-2925, or 33nM NYX-2925 + APV. Sucrose homogenates were fractionated to P2, lysed with 1% Triton-X100 and centrifuged at 32kxg for 20min to separate soluble and insoluble fractions. **A)** Representative Western blots of GluA1 and PSD95 from experimental groups (I= Input, S= Soluble, P=pellet). **B)** Ratiometric analysis of insoluble (pellet) to total (input) GluA1 for experimental groups. N=5 independent experiments, *p<0.05 vs. untreated, **p<0.05 vs. cLTP.

Molecular replacement of NR2B with NR2B-R393A



NR2B siRNA constructs were co-transfected with NR1 and NR2B into HEK293 cells for 24h. Triton extracts were analyzed by Western blot for relative NR2B expression and knock-down. **A)** Representative blots of NR2B and eGFP from siRNA constructs analyzed. **B)** Ratiometeric data of NR2B/eGFP for siRNA constructs, n=3. **C)** The target sequence of TF2 within NR2B was replaced with silent mutations to generate a TF2 siRNA-resistant NR2B with and without the R393A point mutation (WT2B & PM2B, respectively). Note the ineffectiveness of TF2 to knock-down siRNA-resistant NR2B.

NR2B-R393A ablates NYX-2925 potentiation of structural plasticity



Molecular replacement of endogenous NR2B with wild-type NR2B (WT2B) or NR2B-R393A (PM2B) was performed at DIV14 in primary cortical neurons and experiments executed at 48-72h post transfection. **A)** TF2 harboring GCaMP6f, WT2B or PM2B, and dsRED were co-transfected and assayed for sLTP via HFU using the protocol described in Fig 1. **B)** TF2-GCaMP6f and WT2B or PM2B were co-transfected and assayed for cLTP-dependent changes in spine volume (see Methods).