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TITLE: Comparison of Novel and Known Neuroprotectants for Treating Exposure to Different Types of Toxins

PRINCIPAL INVESTIGATOR: Ben A. Bahr, Ph.D.

CONTRACTING ORGANIZATION: University of Connecticut Storrs, Connecticut 06269-1133

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Hippocampal slice cultures were used to examine excitotoxic responses to AMPA and the neurotoxin trimethyltin (TMT) known to cause seizures and hippocampal damage. Relatively short exposure to AMPA and extensive infusion of TMT caused spectrin fragmentation and synaptic deterioration that persisted after a 24-h recovery period. Several avenues of protection were effective at promoting recovery when initiated post-insult. These include anti-inflammatory agents, an anticonvulsant, and modulators of AMPA receptors (Ampakines) that enhance excitatory communication. For example, 20-100 μM Ampakine applied after a 5-h TMT insult decreased the cytoskeletal damage and synaptic decay in a dose-dependent manner. Ampakine also was protective in slices exposed 15 min to AMPA, reducing spectrin breakdown (-81%, p<0.001), presynaptic decay (-77%, p<0.001), and CA1 cell loss (-75%, p<0.01). Neuronal subfields exhibited normal density and morphology 10 days post-insult. To confirm protection in vivo, intrastriatal AMPA injection caused extensive lesion formation and this was reduced by co-injecting Ampakine. Changes in two neuronal markers also indicated protection. Interestingly, Ampakine neuroprotection corresponded with the drug’s positive influence on MAP kinase and NF-κB activation. Moreover, additive effects between different protection avenues were occasionally evident, maximizing the cellular capacity for self-repair when induction of the neuroprotective signaling pathways was not saturated.
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4. INTRODUCTION

The purpose of this research was to identify cellular repair mechanisms that are able to counteract the effects of neurotoxicants that do not involve blockage of important transmitter receptors or signal transduction systems. In the first year of the project, two different neurotoxins were used that have distinct actions of toxicity: 1) the excitotoxin AMPA which causes cytoskeletal damage (Bahr et al., 1995b) and selective cell loss in the hippocampal subfield CA1 (Bahr et al., 2000 – in Appendix), and 2) the toxic pesticide trimethyltin (TMT) which causes seizure activity and selective cell loss in subfield CA3 (see Munirathinam and Bahr, 2000 – in Appendix). With two differently acting toxicants, tests were conducted to assess whether distinct avenues of neuroprotection have similar effects regardless of the mechanism of toxicity. This is important ground work to establish before tests begin on the military threat agent soman since soman appears to have a unique action which leads to persistent damage in multiple hippocampal subfields (Kadar et al., 1992). Moreover, while a correlation between induced convulsions and decreased cell survival was evident in dissociated cell cultures (Deshpande et al., 1995), the in vivo degenerative events associated with soman exposure may be due to a secondary effect(s) unrelated to the agent’s clinical toxicity (Kadar et al., 1992). Accordingly, organotypic hippocampal slice cultures were used in the present report in order to study toxic responses of adult brain tissue. The cultured slices possess many adult features with respect to cellular and synaptic density (Bahr et al., 1995a), circuitry (Bahr, 1995), and pathogenic responsiveness (Bahr et al., 1994, 1995b, 1998). Comparisons were made between the protective actions of three different classes of compounds: antiinflammatory drugs, anticonvulsants, and a novel group of neuroprotectants consisting of glutamatergic potentiator agents (Ampakines). Ampakines improve memory encoding in both rodents and humans, and they also improve repair potential following excitotoxic insults perhaps by augmenting signaling events underlying cellular protection cascades (Bahr et al., 2000). Further studies addressed whether common signaling pathways are involved in the individual avenues for neuroprotection. These studies should establish the potential of combination therapies for treating exposure to neurotoxins as well as neurodegenerative diseases.

5. BODY

Studies were conducted to compare the action of two known classes of protective drugs, i.e. an antiinflammatory drug (acetylsalicylic acid, ASA) and an anticonvulsant drug (huperzine A), with that of a novel neuroprotectant (Ampakine) in a model of excitotoxicity using hippocampal slice cultures. Ampakines cause the positive modulation of AMPA receptors, a subclass of glutamate receptors which mediate many physiological processes via their ionotropic activity but also act as signal transducers with linkages to mitogen-activated protein kinase (MAPK), Src-family kinases, and G-proteins. The first set of experiments set out to establish the neuroprotective potential of Ampakines by testing for their link to cellular repair mechanisms. In long-term hippocampal slices, 0.5-2-min exposure to the agonist AMPA caused the rapid activation of MAPK measured as the phosphorylation of ERK1/ERK2 on Thr183 and Tyr185. This activation event was blocked by the AMPA receptor antagonist CNQX but not by the AP5 antagonist of another class of glutamate receptor, the NMDA receptor (Fig. 1A). While AMPA was used here to show AMPA receptor stimulation is linked to the MAPK pathway, it did not require extensive exposure of the agonist which would cause excitotoxicity. As a related control, the immunoreactivity levels of the phosphorylated MAPK isoforms were increased 2-3-fold,
Figure 1. AMPA receptors are linked to the MAPK pathway. A, AMPA (100 μM) applied to hippocampal slice cultures caused activation of MAPK as assessed with antibodies specific for phosphorylated ERK isoforms on Thr\(^{183}\) and Tyr\(^{185}\), and this activation was blocked by CNQX but not by AP5. B, The Ampakine CX516 (100 μM) applied for 60 min caused MAPK activation in the absence but not presence of the MEK inhibitor PD98059. C, Immunoblots were assessed for activated ERK2 in slices treated with Ampakine, AMPA, or the two together. Integrated optical densities are expressed as means ± SEM, measured from 6-10 blot samples each. Statistics used student t-tests comparing to control: *p = 0.02, ***p = 0.0001, or comparing to AMPA-treated slices: ++p = 0.01.
while total ERK levels were unchanged (not shown; see Fig. 1B). Interestingly, MAPK was activated by the Ampakine CX516 (p = 0.0001; see Fig. 1B and 1C) and the AMPA-induced MAPK activation was further enhanced by the Ampakine (p = 0.01; see latter two bars in Fig. 1C). The allosteric modulation of AMPA receptors appears to mediate the phosphorylation of MAPK by causing the activation of MAPK kinase (MEK), as indicated by the effect of the MEK inhibitor PD98059 (Fig. 1B).

Immunocytochemistry with antibodies selective for phosphorylated MAPK indicate that the induction mediated by AMPA is primarily localized to pyramidal neurons in field CA1 (see arrows in Fig. 2b) as compared to control slices (Fig. 2a, 2d). When Ampakine was present to positive modulate the activity of AMPA receptors during agonist stimulation, the immunostaining of phosphorylated MAPK was more pronounced in the CA1 pyramidal zone and associated dendritic field (Fig. 2c, 2f). Ampakine alone also caused cytoplasmic activation of MAPK in pyramidal neurons (Fig. 2e). In addition to the link to MAPK pathway, recent preliminary data indicate that Ampakine’s influence on MAPK leads to the nuclear translocation and DNA binding of the transcription factor NF-κB (Bahr et al., 2000), a factor believed to promote the expression of a variety of survival genes.

With a link between AMPA receptors and neuroprotective pathways established, it is predicted that positive modulation of the excitatory communication mediated by these receptors will promote survival following cellular injury. Accordingly, an excitotoxic level of AMPA was infused into slice cultures for 15-20 min, resulting in global depolarization, cytoskeletal breakdown, and synaptic deterioration as previously reported (Bahr et al., 1995b). CX516 (1-(quinoxalin-6-ylcarbonyl)piperidine) and other Ampakines were screened for protective effects in the slice model of excitotoxicity. CX516, the Ampakine shown to be active in human memory studies, reduced the level of cytoskeletal proteolysis as measured with antibodies that selectively identify spectrin fragmentation. The amount of spectrin breakdown product (BDP) present 24-36 h after the 15-min excitotoxic insult was reduced by 81% (p<0.001; n=9 immunoblot samples), in a dose-dependent manner. As expected, the degree of cytoskeletal damage in different hippocampal subfields determined the relative vulnerability to cell death following the excitotoxic episode. That is, field CA1 exhibited the most spectrin BDP accumulation, and also expressed significant cell loss compared to the little change in neuronal density in CA3 and dentate gyrus (Fig. 3). CX516 produced cytoskeletal protection and enhanced cellular survival. The number of CA1 cell bodies and dendritic puncta immunostained for spectrin BDP were both markedly decreased by CX516 (Fig. 4). By counting CA1c pyramids within 6.5 x 10^4 μm² fields in control, insult, and insult + drug slices (n=8-16 each), the Ampakine was also found to attenuate neuronal loss and the appearance of pyknotic nuclei by ~75% (p<0.01). A close relative of CX516, 1-(quinoxalin-6-ylcarbonyl)-1,2,3,6-tetrahydro-pyridine (CX515), was used to show that Ampakine applied after an insult provided similar protection (75-100% recovery) from spectrin breakdown and presynaptic loss (p=0.001; n=8) as when pre-incubated. In addition, neuroprotection activity corresponded with the modulatory activity targeting AMPA receptor currents. Derivatives of CX516 that are 10-10,000 times more potent at enhancing monosynaptic EPSPs were tested for effects on spectrin BDP accumulation and synaptic pathology. The protection elicited by 100 μM CX516 was matched by 1 μM CX672, and by 0.5 μM CX691. Regarding the latter, the reduction in spectrin fragments was evident 24 h after the insult (-66%, p=0.02), as well as at 1-2 h post-insult (-98%, p=0.002; n=6-11). The most potent drug, CX697, reduced the cytoskeletal decay measured 20 h post-insult by 50% at 30 nM (p=0.028; n=6). These data indicate that Ampakines are neuroprotective at low concentrations.
Figure 2. MAPK activation by AMPA and Ampakine. Hippocampal slice cultures were untreated (a, d), incubated with 100 μM AMPA for 3 min (b), with 100 μM Ampakine for 5 min (e), or with both AMPA and Ampakine (c, f). The slice cultures were stained with antibodies specific for phosphorylated ERK isoforms on Thr183 and Tyr185, and photomicrographs were prepared at low (a-c) and high (d-f) magnification. sg, stratum granulosum; sp, stratum pyramidale; sr, stratum radiatum.
Figure 3. Groups of 8-16 cultured slices were either not treated or subjected to a 17 minute AMPA insult in the absence or presence of 100μM CX516 (initiated 2 hours prior). Data obtained from tissue fixed and Nissl stained. Cell counts and BDP immunostaining determined utilizing the Bioquant data acquisition system. ANOVA: p=0.01
Figure 4. Recovery from excitotoxin-induced cytoskeletal damage is promoted by Ampakine. Hippocampal slice cultures were either not treated or subjected to a 15 minute AMPA insult in the absence or presence of 100 μM CX516 (initiated 2 hours priors). Slices were fixed 36 hours post-insult, then sectioned and immunostained for calpain-mediated spectrin cleavage. Low-power photomicrographs shown in left panels, high power magnification of CA1 subfield depicted on right. sp, stratum pyramidale; sr stratum radiatum.
Spectrin BDP immunostaining in CA1 was assessed by threshold digitization since this subfield is known to be selectively vulnerable to excitotoxicity (Bednarski et al., 1995; Bahr, 2000). As shown in recent analyses, the Ampakine CX516 markedly reduced the extent of persistent cytoskeletal damage evident a day post-insult; this occurred in the glutamate receptor-rich dendritic zone of the stratum radiatum (-53%; p=0.03) as well as in the stratum pyramidale cell layer (-46%; p<0.01). Preliminary evaluations of subfields CA3 and dentate gyrus also indicate Ampakine-mediated protection although the number of slice samples in the initial study was too low to confirm statistical significance (see Fig. 3). BDP staining in control slices was essentially background which is subtracted from all slice groups. The cytoskeletal breakdown signal demonstrates the powerful sensitivity obtained when combining an in vitro model without systemic variables with the measurement of a pathogenic marker with little to no background signal. Note that a series of in vitro and in vivo studies have shown that similar fragmentation events coincide with early pathogenesis well in advance of any overt cellular atrophy or degeneration (reviewed in Bahr, 1995). The slice model also exhibits classical ischemic-type damage when subjected to excitotoxins (Bahr, 1995; Bahr et al., 1995b; Bednarski et al., 1995). Thus, the in vitro analyses shown in this report are a valuable compliment to in vivo studies.

To confirm that the neuroprotective action of Ampakine found in the slice model also occurs in vivo, we used intrastriatal AMPA injections. In adult Sprague Dawley rats, 50 nmoles of AMPA was injected into the right striatum and found to produce sizeable lesions measured by MRI in vivo 24 h later (Fig. 5b, 5d); control studies showed no such neuronal damage associated with injection of CX516 alone (Fig. 5a, 5d). The Ampakine was then co-injected with the excitotoxin in order to test for protection. As shown in Figure 5e, the lesion extent was found to be attenuated 65% by CX516 in a dose-dependent manner (ANOVA: p=0.02). The Ampakine-mediated neuroprotection was corroborated postmortem by measuring two important neuronal marker enzymes. By within-animal comparison to the non-lesion side of the brain, choline acetyltransferase (CAT) activity exhibited a significant reduction of ~50% (p<0.01) in the striatum as well as in cortical tissue affected by the feed-forward spread of AMPA-induced excitotoxicity (see top panels in Fig. 6). The decrease in CAT activity was attenuated by increasing amounts of CX516 in the striatal (ANOVA: p<0.0001) and cortical regions (p<0.001) as shown in Figure 6. In the lower panels of Figure 6, similar results were found for glutamate decarboxylase (GAD) activity where CX516 elicited protective effects in both striatal (ANOVA: p<0.0001) and cortical areas (p<0.01). Note that while the Ampakine provided complete protection of the two enzymatic activities in the lesioned striatum, it was less effective due to dilution after diffusion to reach the surrounding cortex. The results indicate that AMPA receptors trigger neuroprotective signaling cascade(s) through their excitatory communication, thus to enhance the cellular capacity for self-repair and to counterbalance their own excitotoxic actions.
Figure 5. Protection against AMPA-induced neurodegeneration in vivo by CX516. Lesion induction detected by MRI 24 hours after injection of CX516 (a), AMPA (b) or AMPA with CX516 (c) into the striatum of adult rat brains. Quantitation of lesions (µl) induced by AMPA or CX516 only (d) or with increasing amounts of CX516 (e). *p = 0.02
Figure 6. The activities of CAT (top panels) and GAD (bottom panels) were measured post mortem in two brain regions of the same animals depicted in Figure 5. The levels of CAT and GAD are expressed as the percent decrease in the lesioned vs. non-lesioned side. **p<0.01; ***p<0.001.
Next, tests were conducted to assess whether distinct avenues of neuroprotection have similar effects on one type toxicity, i.e., AMPA-induced excitotoxicity. Accordingly, hippocampal slice cultures were treated with AMPA for 20 min. After the excitotoxic response was briefly quenched, potential protective action was assessed for three different classes of compounds (Ampakines, antiinflammatory drugs, anticonvulsants) when administered during the 24-h recovery period. As described earlier, Ampakine application was just as neuroprotective when administered to slices after the insult vs. before (75-100% cytoskeletal and synaptic recovery). Similarly, NS398, a cyclooxygenase-2 inhibitor which reduces the generation of reactive oxygen species, caused protection of both pre- (synaptophysin; p < 0.05) and postsynaptic markers (GluR1 subunit of AMPA receptors; p = 0.003) 24-h post-insult (Fig. 7). Interestingly, preliminary findings indicate that Ampakine and the antiinflammatory agent act in different ways to enhance repair. The top panel in Figure 7 shows that the Ampakine CX516 has an additive effect in combination with NS398 (p = 0.004). At the early stages of the combination experiments, it is not surprising that we only see significant additive effects on one of the two synaptic markers. It is likely the case that robust protection by the first neuroprotectant will decrease the possibility of a second agent causing further protection as appears to be the case in Figure 7. In initial studies using indomethacin (IND) and acetylsalicylic acid (ASA), these antinflammatory drugs also enhanced recovery to maintain higher concentrations of pre- (p = 0.01-0.03) and postsynaptic (p = 0.02) markers 24 h post-insult as compared to insult only slices (data not shown). When combined with Ampakine, the only evidence of additive effect came from the ASA study where, interestingly, the Ampakine also caused additional enhancement of MAPK activation. Thus, additive effects between different protection avenues allow for the maximizing of cellular repair potential when neuroprotective signaling pathways are not maximally induced.

In order to determine if exposure to differently acting toxicants can be treated with the same protection strategy, tests were conducted to assess whether distinct avenues of neuroprotection have similar effects regardless of the mechanism of toxicity. Accordingly, trimethyltin (TMT), a neurotoxic pesticide known to cause seizures, amnesia, and selective damage to the hippocampus, was used to compare toxic responses to those produced by an AMPA insult. In the long-term hippocampal slice model, TMT (100 μM) was applied for 1-4 h followed by washout and antagonistic quenching of AMPA and NMDA receptors. After a 24-h recovery period, the 1-4 h TMT exposures were associated with increasing levels of calpain-mediated spectrin breakdown and synaptic deterioration. As shown in Figure 8, about the same amount of residual spectrin cleavage products were found accumulated at 5 vs. 24 h post-insult. This is also true with regards to the degree of synaptic damage. Thus, subsequent studies used 4-5 h of TMT exposure in order to induce a sufficient degree of cytoskeletal and synaptic atrophy for recovery analyses, and a 24-h post-insult period was routinely assessed to measure recovery from initial pathogenic events as well as from accumulated pathogenic consequences.

As in the AMPA exposure experiment, Ampakines also exhibited protective effects after the 5-h exposure to TMT. As shown in Figure 9, the Ampakine CX727 acted in a dose-dependent during the 24-h recovery period to reduce spectrin breakdown accumulation, postsynaptic destruction, and presynaptic decay (Fig. 9). Regarding the latter assessment of protection, another Ampakine, CX516, appeared to be more potent that CX727 for promoting maintenance of the presynaptic marker. With further analysis, CX516 was more effective at reducing persistent spectrin fragments after the TMT insult than was CX727 (Figs. 9 and 10). As expected, the anticonvulsant huperzine also was protective against TMT exposure (pilot data).
Figure 7. Control cultured slices (NT) or slices treated with 100 μM AMPA for 20 min, quenched, then allowed to recover in the absence or presence of NS398, Ampakine, or both were assessed for GluR1 and synaptophysin by immunoblot. Student t-test were used to compare the neuroprotection data to AMPA only slice groups; *comparison was to NS398 protection group.
Figure 8. Time course of the neurotoxic response to TMT. Cultured hippocampal slices were treated with 100 µM TMT for different time periods followed by immediate quenching of glutamate receptor subtypes with antagonists. Slices were harvested 5 and 24 h post-insult and assessed for spectrin breakdown product (BDPn) and GluR1 by immunoblot.
Figure 9. Control cultured slices (NT) or slices treated with 100 μM TMT for 4.5 h in the absence or presence of the indicated Amakines were assessed for spectrin breakdown, GluR1, and synaptophysin by immunoblot. Student t-test were used to compare the neuroprotection data with TMT only slice groups.
Figure 10. Control cultured slices (NT) or slices treated with 100 μM TMT for 4.5 h in the absence or presence of the indicated concentrations of Ampakine CX516 were assessed for spectrin breakdown and GluR1 by immunoblot. Student t-test were used to compare the neuroprotection data to TMT only slice groups.
Stable cellular recovery from TMT exposure mediated by CX516 was assessed by Nissl. In contrast to the CA1 vulnerability following exposure to an excitotoxin like AMPA or NMDA (Bednarski et al., 1995; Bahr, 2000), Figure 11 shows that TMT exposure preferentially targets hippocampal field CA3 and, to a lesser degree, the dentate gyrus (DG). The Ampakine protected both subfields targeted with regards to cellular density and morphology (Fig. 12). These results demonstrate that Ampakines offer pronounced neuroprotection against organotin-induced toxicity.

Figure 11. Low power photomicrographs show Nissl stained sections from control (A) and TMT-treated slice culture (B) slice. Arrows indicate dramatic decrease in cell density in the layers of CA3 and DG.
Figure 12. Top Panels, Control slice cultures exhibit normal cellular density in field CA3 (left) and dentate gyrus (right). Middle Panels, Slices treated with TMT exhibit reduced cell density in the two subfields and evidence of pyknotic transformation. Bottom Panel, TMT-treated slices provided with Ampakine CX516 during the 24-h recovery period showed significant protection with regard to cell density, morphology, and degree of pyknotic changes.
6. KEY RESEARCH ACCOMPLISHMENTS

- Established a correlation between activation of a neuroprotective signaling pathway(s) and the enhancement of cellular/synaptic recovery following an insult.

- Determined the localized activation of neuroprotective signaling pathways in order to compare to recovery events in different hippocampal subfields.

- Established that cytoskeletal deterioration is an early response to neurotoxins that determines cellular vulnerability and extent of recovery.

- Established a good correspondence between different recovery markers: cytoskeletal protection, synaptic maintenance, and cell survival.

- Determined that the neuroprotective pathways identified in the *in vitro* slice model are also relevant and effective in *in vivo* studies.

- Established that distinct avenues of protection can counteract the consequences of the same type of excitotoxin exposure.

- Established evidence that two different avenues of protection can together produce recovery in an additive fashion following exposure to an excitotoxin.

- Determined that one particular neuroprotection pathway (i.e. Ampakine therapy) can enhance recovery following the exposure to at least two different types of toxins (the excitotoxin AMPA and the convulsant TMT).
7. REPORTABLE OUTCOMES

- **MANUSCRIPTS** (attached at end of appendices)

- **ABSTRACTS** (included in appendices)
  4. Brown QB and Bahr BA (2000) Phosphorylation of the ERK1/ERK2 MAP kinase can be influenced by AMPA-type glutamate receptors and their positive modulation in hippocampus. *J Neurochem* 74 (Suppl.):S20D.


PRESENTATIONS (included in appendices)


8. CONCLUSIONS

This work provides a positive outlook with regard to identifying potential repair strategies for treating exposure to the neurotoxin soman, and to utilize combination therapies to achieve pronounced additive recovery effects with distinct avenues of protection. With two different toxicants, AMPA and TMT, one being similar to soman in action, multiple strategies of neuroprotection were found to produce comparable enhancement of recovery. The protection was independent of the mechanism of toxicity, this in light of the fact that AMPA and TMT target distinct neuronal populations in the hippocampus. Furthermore, the two different pathogenic processes were reduced in damage potential by the same protection pathway (i.e., mediated by Ampakine and associated influence on MAPK and NF-κB). While common signaling pathways may be involved in the individual avenues for neuroprotection, these studies established the potential of combination therapies for treating exposure to neurotoxins as well as neurodegenerative diseases.

9. REFERENCES

10. APPENDICES

- MANUSCRIPTS (attached at the end)


- ABSTRACTS (pages 26-35)


4. Brown QB and Bahr BA (2000) Phosphorylation of the ERK1/ERK2 MAP kinase can be influenced by AMPA-type glutamate receptors and their positive modulation in hippocampus. *J Neurochem* 74 (Suppl.):S20D.


 CURRICULUM VITAE: Ben A. Bahr (pages 36-52)
Ampakine Modulators of AMPA-Type Glutamate Receptors Promote Neuronal Recovery after Excitotoxic Injury in Brain Tissue.

Ampakines enhance the ionotropic activity of AMPA receptors, glutamate-gated channels also known to function as cell-surface signal transducers by interacting with and/or regulating G-proteins, Src-family kinases, and MAP kinase pathways involving CREB and the neurotrophic factor BDNF (Nature 389:502, 1997; ibid. 397:72, 1999; J. Neuroscience 19:2954&5861, 1999). This study tested whether Ampakines can promote cellular viability by augmenting such "metabotropic" actions. Accordingly, CX516 (l-(quinoxalin-6-ylcarbonyl)piperidine) and other Ampakines were screened for protective effects in hippocampal slice cultures and adult rats treated with excitotoxic levels of AMPA (15-min infusion vs. intrastriatal injection). In the slices, the selective loss of CA1 neurons evident 24 h later was reduced 75% by 100 μM CX516; all subfields exhibited normal cell density and morphology 10 days post-insult.

In the rats, CX516 co-injected with AMPA attenuated the lesion extent by 65% in a dose-dependent manner. Effects on changes in neuronal markers (CAT and GAD) also indicated protection. Persistent injury in the slice model, measured as calpain-induced cytoskeletal breakdown, exhibited Ampakine-mediated recovery in pyramidal and dendritic zones. Such cellular recovery was evident when Ampakines were administered before or after the excitotoxic episode. These data indicate that Ampakines promote signaling cascade(s) underlying neuroprotection. Close correspondence between protective action and electrophysiological influences among micromolar- and nanomolar-potent analogs will be discussed. (supported by the American Federation for Aging Research and U.S. Army Medical Research Grant DAMD17-99-C9090).

J. Bendiske1, G. Rogers2, M. Rudin3, S. Urwyler3, A. Sauter3, and B.A. Bahr1. 1Dept. Pharmaceutical Sciences, University of Connecticut; 2Cortex Pharmaceuticals, Irvine, CA; 3Novartis Pharma AG, Basel, Switzerland.
**Title:** Positive Modulation of AMPA Receptors Promotes Cellular Repair Following Excitotoxic Injury to Brain Tissue in vitro and in vivo.

**Author Block:** B.A. Bahr¹, J. Bendiske¹, G. Rogers², M. Rudin³, S. Urwyler³, A. Sauter³

¹University of Connecticut: Storrs, CT; ²Cortex Pharmaceuticals: Irvine, CA; ³Novartis Pharma AG: Basel, Switzerland

**Abstract Text:**

Ampakines are compounds developed for the treatment of neurological disorders. They enhance the ionotropic activity of AMPA receptors, glutamate-gated channels also known to function as cell-surface signal transducers by interacting with and/or regulating G-proteins, Src-family kinases, and MAP kinase pathways involving CREB and the neurotrophic factor BDNF (Nature 389:502, 1997; ibid. 397:72, 1999; J. Neuroscience 19:2954&5861, 1999). This study tested whether Ampakines can promote cellular viability by augmenting such metabotropic actions. Accordingly, CX516 (1-(quinazolin-6-ylcarbonyl)piperidine) and other Ampakines were screened for protective effects in hippocampal slice cultures and adult rats treated with excitotoxic levels of AMPA (15-min infusion vs. intrastriatal injection). In the slices, the selective loss of CA1 neurons evident 24 h later was reduced 75% by 100 µM CX516; all subfields exhibited normal cell density and morphology 10 days post-insult when the Ampakine was administered. In the rats, CX516 co-injected with AMPA attenuated the MRI-measured lesion extent by 65% in a dose-dependent manner. Effects on changes in neuronal markers (CAT and GAD) also indicated protection. Persistent injury in the slice model, measured as calpain-induced cytoskeletal breakdown, exhibited Ampakine-mediated recovery in pyramidal and dendritic zones. Such cellular recovery was evident when Ampakines were administered before or after the excitotoxic episode. These data indicate that Ampakines promote signaling cascade(s) underlying neuroprotection. Close correspondence between protective action and electrophysiological influences among micromolar- and nanomolar-potent analogs will be discussed.
TAU MODIFICATIONS AND CONCOMITANT TRANSPORT DISTURBANCES FOLLOWING LYSOSOMAL DYSFUNCTION.

Pathogenic modification of tau species leads to paired helical filament (PHF) formation with brain aging, especially in Alzheimer patients, and is thought to result in synaptic dysfunction (Callahan and Coleman, Neurobiol Aging 16:311, 1995). To test whether the link between the tau modifications and synaptic atrophy is caused by transport failure, correlative analyses were carried out in hippocampal slice cultures expressing age-related events. Specifically, slices were treated with the lysosomotropic agent chloroquine (CQN) which can induce many manifestations of brain aging and Alzheimer’s disease. Over the treatment period, time-dependent events included 1) an increase in phosphorylated tau isoforms of 55-69 kDa, 2) a corresponding elevation in cathepsin D levels, and 3) a decline in synaptophysin and its message. The tau changes were associated with gradual intraneuronal deposition of material recognized by antibodies to human PHFs and ubiquitin. The deposits formed as a polar accumulation, concentrated in and around the axon hillock. This would likely influence transport processes and, correspondingly, non-aggregated tau shifted from a normally diffuse to a narrow zonal distribution in dendritic fields of treated slices, indicative of transport disturbances. The CQN-induced modification may keep tau from stabilizing microtubule integrity. Interestingly, the microtubule component α-tubulin expressed a correlative deacetylation, an event also found in Alzheimer brains. To directly evaluate transport, HRP was applied to CA1 pyramids. Control cells exhibited HRP uptake and transport to distal dendrites, whereas CQN-treated slices had somatic HRP staining with increasingly sporadic detection of transport over days of treatment. The findings suggest that age-related modification of tau isoforms disrupts microtubule function, thus blocking transport processes important for synaptic maintenance (supported by the American Federation for Aging Research and U.S. Army Medical Research Grant DAMD17-99-C9090).
PHOSPHORYLATION OF THE ERK1/ERK2 MAP KINASE CAN BE INFLUENCED BY AMPA-TYPE GLUTAMATE RECEPTORS AND THEIR POSITIVE MODULATION IN HIPPOCAMPUS.
Queenie B. Brown and Ben A. Bahr. Department of Pharmaceutical Sciences and the Neurosciences Program, University of Connecticut, Storrs, CT.

In the central nervous system, AMPA-type glutamate receptors mediate a wide range of physiological processes including fast excitatory neurotransmission, synaptic plasticity, and certain forms of memory. While these processes are thought to occur via the ionotropic activity of AMPA receptors, the receptors also have been shown to promote mitogen-activated protein kinase (MAPK) signaling cascades (Wang and Durkin, JBC 270:22783, 1995; Perkinton et al., J. Neuroscience 19:5861, 1999). The present study tested whether AMPA receptors mediate the activation of the ERK1/ERK2 MAPK in long-term hippocampal slices, and whether the MAPK activity state can be influenced by allosteric modulators of AMPA receptors (Ampakines). Brief application of AMPA caused the rapid induction of activated MAPK measured as the phosphorylation of the catalytic core of the ERK enzyme on Thr183 and Tyr85 presumably mediated by MAPK kinase (MEK). The dually phosphorylated active forms of ERK1 (p44) and ERK2 (p42) were enhanced two- to three-fold immediately after the 5-min AMPA treatment, while total levels of the isoforms were not changed. Interestingly, occupation of the Ampakine modulatory site caused similar stimulation of MAPK in the absence of AMPA. The activation response of MAPK mediated by AMPA and Ampakine did not appear to be additive. In addition, prolonged exposure to Ampakine was able to sustain the phosphorylation of the MAPK isoforms for several hours. The link between AMPA receptors and the MAPK signal transduction pathway may explain the neuroprotective action of Ampakines and other positive modulators. This work was supported by U.S. Army Medical Research Grant DAMD17-99-C9090 and NIH Grant 1R43NS38404-01.

Signal transduction
Glutamate
AMPA
Excitatory amino acid
Hippocampus

Ben A. Bahr

Queenie B. Brown

University of Connecticut Bldg U-92
Pharmacy Research Institute
Storrs CT USA 06269
(860)-486-1560 (860)486-6858 queenieb@hotmail.com
MULTIFACETED PROPERTIES OF AMPA RECEPTORS INCLUDE LINKS TO NEUROPROTECTIVE SIGNALING PATHWAYS.

B. A. Bahr¹, J. Bendiske¹, Q. B. Brown¹, G. Rogers², M. Rudin³, S. Urwyler³, and A. Sauter³. ¹Department of Pharmaceutical Sciences and the Neurosciences Program, University of Connecticut, Storrs, CT; ²Cortex Pharmaceuticals, Irvine, CA; ³Novartis Pharma AG, Basel, Switzerland.

AMPA receptors are glutamate-gated ion channels responsible for most of the fast excitatory synaptic activity in the brain. They are the primary element of certain forms of synaptic plasticity and facilitate the action of new pharmaceuticals (i.e., Ampakines) that enhance memory. In addition to their ionotropic properties, the receptors can act as cell-surface signal transducers with linkages to mitogen-activated protein kinase (MAPK) pathways, Src-family kinases, and G-proteins. Using Ampakine-mediated positive modulation, our work found evidence that AMPA receptors, through associated signaling pathways, promote the brain's network remodeling capability for recovery from injury. CX516 and other Ampakines were found to have protective effects both in vitro and in vivo. Hippocampal slice cultures exposed to excitotoxins for 15 min exhibited selective loss of CA1 neurons and synaptic markers. Ampakines significantly reduced the neuropathology evident 24 h after the insult. All subfields in Ampakine-treated slices exhibited stable recovery as cell density and morphology were normal 10 days post-insult. The enhanced recovery did not require Ampakines to be present before the excitotoxic episode, and there was an apparent correspondence between the neuroprotective response and the activation of MAPK. In adult rats, intrastriatal injection of AMPA caused extensive lesion formation measured by MRI. Ampakine co-injected with the excitotoxin reduced the lesion extent and did so in a dose-dependent manner. Effects on changes in neuronal markers also indicated protection. The results indicate that AMPA receptors trigger signaling cascade(s) to enhance the cellular capacity for self-repair and thus counterbalance pathogenic events initiated by their own ionotropic activity (supported by U.S. Army Medical Research Grant DAMD17-99-C9090 and NIH Grant 1R43NS38404-01).

Ben A. Bahr

Bahr  Assistant Professor

University of Connecticut

Pharmacy Research Institute

Storrs CT USA 06269-2092

(860)486-6043 (860)486-6858 Bahr@uconn.edu
EVALUATION OF MITOGEN-ACTIVATED PROTEIN KINASE PATHWAYS IN AN IN VITRO MODEL OF NEUROPROTECTION.

Ebru Caba, Queenie B. Brown, and Ben A. Bahr. Dept. of Pharmaceutical Sciences and the Neurosciences Program, University of Connecticut, Storrs.

When over stimulated, NMDA- and AMPA-type excitatory amino acid receptors can induce global depolarization resulting in excitotoxic damage (J. Pharmacol. Exp. Ther. 273:902, 1995). To survive such insults, cells may employ a variety of signaling cascades involving mitogen-activated protein kinases (MAPKs) (e.g., see Science 286:785, 1999). To evaluate potential neuroprotective signals, slice cultures were used from hippocampus, a region that is selectively vulnerable to stroke-type excitotoxicity. The slices can be maintained in culture for months while expressing stable characteristics of the adult brain. When exposed to an excitotoxin for 5-20 min, the slice cultures exhibited a pathogenic responsiveness similar to that expected from in vivo experiments. Injury was measured as persistent spectrin breakdown mediated by the calcium-activated enzyme calpain. The proteolytic response evident several hours post-insult was attenuated by the selective calpain inhibitor CX295 as were subsequent declines in presynaptic markers. Besides disrupting calpain signals, we tested whether the neuroprotectant action of CX295 influences signaling pathways involving three members of the MAPK superfamily. Brief stimulation of AMPA receptors caused rapid induction of activated ERK MAPK, as measured by the presence of a dually phosphorylated catalytic core on Thr and Tyr residues, but no apparent activation of p38 MAPK or c-Jun N-terminal protein kinase (JNK). The active forms of ERK1 and ERK2 were enhanced two- to three-fold by the AMPA treatment, whether it occurred before or several hours after an excitotoxic insult. When CX295 was present during the recovery period, AMPA induction of ERK1/ERK2 appeared to be greater than before the insult. CX295 did not cause activation of the MAPK in the absence of synaptic stimulation as occurs with another class of neuroprotection (see Brown and Bahr abstract). This work was supported by U.S. Army Medical Research Grant DAMD17-99-C9090 and NIH Grant 1R43NS38404-01.
POSITIVE MODULATION OF HIPPOCAMPAL AMPA RECEPTORS PROMOTES RECOVERY SEVERAL HOURS AFTER TRIMETHYL Tin-INDUCED EXCITOTOXICITY.

S. Munirathinam; B.A. Bahr
1. Pharm. Sci./Neurosciences, Univ. of Connecticut, Storrs, CT, USA

AMPA receptors mediate a wide range of CNS processes including fast excitatory transmission and memory-related plasticity. While these events occur mainly via ionotropic activity, the receptors also have been shown to promote neuroprotective signaling cascades. The present study tested whether such metabotropic action can be tapped by allosteric modulators of AMPA receptors (Ampakines) in order to promote self-repair following trimethyltin (TMT) exposure. TMT, a neurotoxic pesticide, is known to cause seizures, amnesia, and selective damage to the hippocampus. In the long-term hippocampal slice model, TMT (100 μM) was applied for 1-5 h followed by washout and antagonistic-quenching of AMPA and NMDA receptors. After a 24-h recovery period, the TMT exposures were associated with increasing levels of calpain-mediated spectrin breakdown and synaptic deterioration. Interestingly, the Ampakine CX516 acted during the recovery period to reduce the two events of neurodegeneration. That is, 20 μM CX516 applied after a 5-h TMT insult decreased the cytoskeletal damage and synaptic decay evident 24 h later, and 100 μM of the Ampakine further reduced the pathogenic markers. Stable recovery with regards to cell density and morphology was assessed by Nissl. These results demonstrate that Ampakines offer pronounced neuroprotection against organotin-induced excitotoxicity.
LINKS TO METABOTROPIC SIGNALING ALLOW AMPA RECEPTORS TO COUNTERACT THE NEURODEGENERATION RESULTING FROM THEIR OWN IONOTROPIC OVER-ACTIVITY.

B.A. Bahr*; J. Bendiske1; Q.B. Brown1; E. Caba1; G. Rogers2; M. Rudin3; S. Urwyler3; A. Sauter1

1. Dept of Pharm Sci/Neurosciences Program, Univ of Connecticut, Storrs, CT, USA
2. Cortex Pharmaceuticals, Irvine, CA, USA
3. Novartis AG, Basel, Switzerland

AMPA receptors mediate many physiological processes via their ionotropic activity. In addition, the receptors can act as signal transducers with linkages to mitogen-activated protein kinase (MAPK), Src-family kinases, and G-proteins. In long-term hippocampal slices, 0.5-2-min exposure to AMPA caused the rapid activation of MAPK measured as the phosphorylation of ERK1/ERK2 on Thr183 and Tyr185. The phosphorylated isoforms were increased 2-3-fold, while total ERK levels were unchanged. The induction of MAPK was further enhanced by Ampakines, allosteric modulators of AMPA receptors, and this was associated with distinct changes in NF-kB activity. Interestingly, Ampakines also promoted recovery from injury. Cultured slices exposed to excitotoxins for 15 min exhibited loss of CA1 neurons and synaptic markers. CX516 and other Ampakines acted during the recovery period to reduce the neuropathology evident 24 h after the insult. Neuronal subfields exhibited stable recovery as cell density and morphology were normal 10 days post-insult. In rats, intrastriatal injection of AMPA caused extensive lesion formation. Ampakine co-injected with the excitotoxin reduced the lesion extent in a dose-dependent manner. Effects on changes in neuronal markers also indicated protection. The results indicate that AMPA receptors trigger neuroprotective signaling cascade(s) to enhance the cellular capacity for self-repair and thus counterbalance their own excitotoxic actions.

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"INTERACTION BETWEEN AMPA RECEPTOR CHANNELS: A MECHANISM FOR MODIFYING SYNAPTIC STRENGTH"

V. Suppiramaniam1*, K. Manivannan2; T. Subramaniam1; A. Dityatev3; B.A. Bahr4

1. Dept. of Biology, Tuskegee University, AL,
2. Southwest Missouri State University, Physics, Astronomy & Mat. Sci., Springfield, MO,
3. Institut fuer Biosynthese Neuraler Strukturen, Zentrum fuer Molekulare Neurobiologie Hamburg, Univ. of Hamburg, Hamburg, Germany
4. Dept. of Pharmaceutical Science, Univ. of Connecticut, Storrs, CT,

Interactive gating behavior of ion channels has been reported in a wide variety of channel types such as gap junction channels, acetylcholine channels, and AMPA receptor channels. Recently, AMPA receptors have been shown to cluster at the postsynaptic domain during tetanic stimulation. Furthermore, alteration of AMPA receptor channel kinetics has been implicated in the expression of long term potentiation (LTP). Our recent findings suggest that the close proximity of AMPA receptor channels due to clustering may give rise to lateral interactions between receptors leading to cooperative channel gating (Suppiramaniam, J. Neurochem. 74:S50A, 2000). We have utilized purified and immunoprecipitated AMPA receptors reconstituted in lipid bilayers to demonstrate such interactions in the presence of proteoglycan components that are endogenous to synapse and a cognitive enhancer ampakine. All these compounds at nanomolar concentrations have been previously shown to interact with AMPA receptors (Bahr et al., J. Mol. Biol. Biochem. 1:35, 1999). However, the details of the mechanism of induction of interactive channel gating behavior of AMPA receptors by these molecules have not been well understood. We present an analysis of multi-channel data from AMPA receptor channels using previously reported model-independent methods (Manivannan et al., Biophys. J. 61:216, 1992). First, we test for independent gating behavior by analyzing the all-points current amplitude histograms. Then, we utilize a simple steady-state model that involves only two parameters to characterize the interactive gating behavior of AMPA receptor channels (Manivannan et al., Bull. of Math. Biol. 58(1): 141, 1996). Using current amplitude histograms we extract these two parameters which measure the open probability of single channels and the "strength" of interaction between channels. Quantitative analysis revealed that the endogenous components and ampakine strongly enhanced the interactive channel gating of AMPA receptors with long-lived substates. These findings indicate that interactive channel gating among AMPA receptors may be a potential mechanism that modifies synaptic efficacy.

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TAU DEPOSITION IS LINKED TO MICROTUBULE DESTABILIZATION AND CONCOMITANT SYNAPTIC DECLINE.

J. Bendiske; E. Caba; S. Lan; G. Gianutsos*; B.A. Bahr

1. Dept. of Pharm.Sci./Neurosciences Program, Univ. of Connecticut, Storrs, CT, USA

Aged brains, especially those of Alzheimer patients, exhibit a correlation between lysosomal perturbation and synaptic decay. Using the long-term hippocampal slice model, mild lysosomal dysfunction mediated by chloroquine (CQN) was tested for corresponding changes in tau, microtubule stability, and synaptic markers. After several days, an increase in hyperphosphorylated tau species occurred prior to the intraneuronal deposition of material recognized by antibodies to human PHFs and ubiquitin. The PHF-like deposits exhibited a basal orientation within pyramidal cells, thus, blockage of axonal and basal dendritic transport could account for the decline in synaptic markers found associated with lysosomal dysfunction. Correspondingly, several events also occurred that indicate microtubule destabilization including 1) gradual decline in acetylated tubulin, an event also noted in Alzheimer brains, 2) concurrent production of tubulin fragments, 3) abnormal localization of non-phosphorylated tau species, and 4) severe disturbances in transport assays using HRP. The latter included a conspicuous lack of transport of HRP in both apical and basal directions, despite obvious perikaryal uptake. This indicates that CQN-induced decline in synaptophysin protein and mRNA is due to disruption of microtubule function rather than a physical blockage. Thus, events that promote tau deposition and the loss of normal tau function also disrupt transport mechanisms important for the maintenance of central synapses.

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CURRICULUM VITAE: Ben A. Bahr

CURRENT ADDRESS
Dept. of Pharmaceutical Sciences & the Neuroscience Program
University of Connecticut
Storrs, Connecticut 06269-2092
tel 860-486-6043, fax 860-486-6858, Bahr@uconn.edu

DEGREES
1984 B.A., Biochemistry & B.A., Molecular Biology Department of Biological Sciences
University of California, Santa Barbara, California
1989 Ph.D., Chemistry Department of Chemistry (with the Neuroscience Research Institute)
University of California, Santa Barbara, California

POSITIONS
1997 - Assistant Professor of Pharmacology and Neurotoxicology, Dept. of
Pharmaceutical Sciences, University of Connecticut, Storrs, CT.
1995-1996 Associate Researcher I, Center for the Neurobiology of Learning and Memory,
University of California, Irvine (UCI). Center Director: Dr. James L. McGaugh.
1990-1994 Lecturer, Department of Psychobiology, UCI. Department Chair: Dr. Killackey.
1989-1995 NRSA Fellow, Assistant Researcher I/II; UCI, under Drs. Lynch and Cotman.

DISTINCTIONS
The Professor B.R. Baker Memorial Award and Fellowship in Chemistry, 1987
University of California General Affiliates Fellowship, 1988
The NATO and FEBS Advanced Study Institute Fellowship, 1988
National Science Foundation International Travel Award, 1988
The American Chemical Society Pacific Division Invited Keynote Address, 1991
Young Investigator Award from the University of California, Irvine, 1995
Faculty Career Development Award from the University of California, Irvine, 1996
Colloquium Organizer and Chair for the American Society for Neurochemistry, 2000
Young Investigator Award from the International Society for Neurochemistry, 2000
Editorial Board Member, Journal of Molecular Biology and Biotechnology, 2000 -

PROFESSIONAL AFFILIATIONS
Society for Neuroscience, Member since 1985
American Society for Cell Biology, Member since 1990
American Society for Pharmacology & Experimental Therapeutics, Member since 1995
Society of Toxicology, Member since 1998
American Society for Neurochemistry, Member since 1998

36
PATENTS
2000  “Cellular Regulation to Suppress Neuropathogenesis” (UConn Invention #00-004)

RESEARCH INTERESTS
Links Between Receptor Signaling Events, Synaptic Regulation, and Cellular Repair
Neuroprotection in Models of Alzheimer's Disease, Brain Aging, and Stroke
Pathogenic Cascades Involving Aberrant Protein Processing and Synaptic Failure

PROFESSIONAL ACTIVITIES
1989-93  Organization Committee Member of the Human Frontiers Science Program, CNLM, University of California
1990-91  Member of the Health Sciences Advisory Committee, School of Biological Sciences, University of California, Irvine (UCI)
1990-92  Chair of the Hebb Seminars, CNLM, University of California, Irvine
1990-92  Research Advisor: The Gerard Award Program, Dept. of Psychobiol., UCI
1990-92  Member of the Seminar and Workshop Program Committee, CNLM, UCI
1992-93  Research Advisor: The Dean's Research Award Program, Medical Education College of Medicine, University of California, Irvine.
1992-95  Organization Committee Member of the Irvine Institute for Brain Aging & Dementia, University of California, Irvine
1992-93  Research Advisor: National Science Foundation Research Experience for Undergraduates Program, Dept. of Psychobiology, Univ. of Calif., Irvine
1992-94  Advisor for the President's Undergraduate Research Fellowship, School of Biological Sciences, University of California, Irvine
1993  Research Proposal Reviewer, NSF Program of Neuroscience
1993-  Peer Review Consultant, National Institutes of Health / ADAMHA
1993-94  Research Advisor: Instructional Development Research Fellowship Program, UCI
1995-  Reviewer for J Neuroscience; J Comparative Neurology; J Neurobiology of Learning and Memory; J Neurochemistry; Hippocampus; Neuroscience
1996-97  Chair of the Institutional Animal Care & Use Committee (IACUC), Cortex Inc.
1998  Member of the Pharmacol. Faculty Search Committee, University of Connecticut
1998-  Program Member for Auburn Universities Neuroscience Conference Series
1999-00  Symposium Program Committee, American Society for Neurochemistry Meeting
2000  Co-Chair of the Pharmacology Faculty Search Committee, Univ. of Connecticut
2000  Organizer and Chair of the Annual Hewitt Symposium, University of Connecticut
INVITED SEMINAR PRESENTATIONS

1989, University of Washington Department of Physiology and Biophysics Seminar, "Copurification of the Vesamicol Receptor and the SV1 Antigen of Torpedo Synaptic Vesicles: Is the Acetylcholine Transporter a Proteoglycan?", Seattle, Washington.

1989, University of California Molecular Biology and Biochemistry Section Seminar, "Neurotransmitter Storage Properties in Cholinergic Neurons", Santa Barbara, California.

1990, Academy of Sciences Institute of Neurobiology and Brain Research Seminar, "Transmembrane Linkages Between the Extracellular Matrix and the Cytoskeleton Affecting Long-Term Synaptic Modulation", Magdeburg, East Germany.

1990, Univ. of California Neuroscience Research Seminar, "Identification of Synaptosomal Adhesion Components and their Role in Neuronal Plasticity", Santa Barbara, California.

1990, Univ. of California Medical Center Seminar, "Disassembling and Reassembling Synapses: Points of Contact Between Plasticity and Pathology", City of Orange, Calif.


1994, Univ. of California Learning and Memory Seminar, "Alternative Approaches Toward the Understanding of Aging Processes that Debilitate Brain Function: Development of an Aging Model Using Hippocampal Slice Cultures that Can Express Cellular Events Prominent in the Aged Human Brain and Alzheimer's Disease in Days Instead of Decades", Irvine, CA.


1999, University of Connecticut Department of Psychology: "Development of a Model System to Study Age-Related Processes that Lead to Synaptic Pathology", Mansfield-Storrs, Connecticut.


2000, Nathan Kline Institute/New York University Medical School Seminar: "Abnormal Protein Processing is Linked to Microtubule Destabilization and Concomitant Synaptic Decline", Orangeburg, New York.


ABSTRACTS


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Structural Chemistries Underlying Synaptic Signaling and Regulation

Ben A. Bahr¹, Solomon Yilma², and Vishnu Suppiramaniam²

¹Department of Pharmaceutical Sciences and the Neurosciences Program
University of Connecticut, Storrs, CT 06269-2092

²Department of Biology
Tuskegee University, Tuskegee, AL 36088

Correspondence: Ben A. Bahr, Department of Pharmaceutical Sciences, University of Connecticut, Storrs, CT 06269-2092 (tel: 860-486-6043; fax: 860-486-4998; e-mail: Bahr@uconn.edu)

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ABSTRACT

Structural elements required for cell-cell interactions (integrins, NCAMs, cadherins) and assembly of pericellular matrices (fibronectin, proteoglycans) have been the focus of recent studies to assess their respective roles in the functional regulation of central synapses. The findings indicate that adhesion and matrix molecules involved in membrane conformation play a significant role in the structural basis for learning and memory, an idea which evolved from the strong link between network remodeling and morphological changes in dendritic spines. Defining the complex interplay between the extracellular matrix and cell surface constituents will be necessary to understand how constraints in membrane organization and architecture translate into stable regulation of synaptic signaling.

Adhesion molecules and cell surface proteoglycans have been increasingly implicated in the structural modifications associated with synaptic plasticity. It was in 1893 when Tanzi first speculated that structural changes provide an adequate explanation for the otherwise mysterious persistence of memory. The idea that structural elements are important for plasticity mechanisms underlying learning and memory was proposed 50 years ago (Hebb, 1949), it matured with the discovery of postsynaptic morphological alterations (Lee et al., 1980; Desmond and Levy, 1983; Chang and Greenough, 1984; Geinisman et al., 1993; Buchs and Muller, 1996; Comery et al., 1996; Durand et al., 1996), and it has gained further support from advances in the understanding of adhesion and extracellular matrix chemistries. Though many laboratories have examined elements of adhesion and the membrane cytoskeleton for their roles in learning processes, this review will focus on reports linking the action of structural components and the stable regulation of neurotransmission. In particular, those studies measuring the long-term potentiation (LTP) of glutamatergic responses in hippocampal CA1 will be compared. Such stable enhancement of synaptic efficacy has been suggested to involve a number of distinct structural components including 1) integrin-type matrix receptors, 2) extracellular matrix proteins recognized by integrins, 3) neural cell adhesion molecules of the Ig family (NCAMs), 4) members of the cadherin family of adhesion molecules,
and 5) heparan sulfate proteoglycans.

**Integrin-Type Matrix Receptors**

Integrins are transmembrane matrix receptors whose adhesion responses are known to regulate intracellular signal transduction pathways including those that involve tyrosine, serine/threonine, and mitogen-activated protein kinases (reviewed by Clark and Brugge, 1995; Jones, 1996). Interactions between cell-surface constituents and the extracellular matrix are critical for membrane organization and morphology (Horwitz et al., 1986; Clark and Hynes, 1996), thus matrix receptors were proposed a decade ago as candidates for stabilizing the structural changes associated with synaptic plasticity (Bahr, 1989). Accordingly, integrin-type matrix recognition sites were shown to be required for the stable establishment of hippocampal LTP (Staubli et al., 1990, 1998; Xiao et al., 1991; Bahr et al., 1997). Peptide antagonists were used containing the Arg-Gly-Asp-Ser (RGDS) matrix domain targeted by most integrins (Pierschbacher and Ruoslahti, 1984). The peptides exhibited a similar selectivity for blocking LTP as for disrupting cell-cell and cell-matrix interactions, and their destabilization effects occurred without disrupting the complex physiological responses to LTP induction stimuli or the initial degree of potentiation (Xiao et al., 1991; Bahr et al., 1997). In addition, there was a correspondence between the dose requirement for the LTP effect and that for suppression of integrin-mediated adhesion (Plow et al., 1985; Ruoslahti and Pierschbacher, 1987). A related report demonstrated that RGDS peptides disrupt the expression of kindled seizures in hippocampal slices, thus providing another example of integrin-mediated physiological stability (Grooms and Jones, 1997). Moreover, integrin-related chemistries have been implicated in mechanisms of memory in rats and Drosophila (Grotewiel et al., 1998; Huang et al., 1998).

The pharmacological studies strongly suggest a role for integrins in synaptic regulation, one that may entail enhanced expression of integrin genes (see recent study by Pinkstaff et al., 1998). While integrin-type adhesion receptors are involved in brain development, surprisingly little is known about the functions of those matrix receptors continued to be expressed in the adult brain and, in many instances, localized to synaptic contacts (Grooms et al., 1993; Einheber et al., 1996; Pinkstaff et al., 1998, 1999). Synaptic adhesion experiments demonstrated that forebrain synaptosomes adhere to extracellular matrix proteins via the RGDS integrin target sequence (see Bahr and Lynch, 1992). Follow up studies isolated distinct proteins found concentrated in the synaptic plasma membrane (SPM) fraction which not only bind the RGDS domain of matrix components but also possess integrin epitopes (Bahr et al., 1991, 1997; Bahr and Lynch, 1992). The integrin-like, synaptic matrix receptors identified (i.e., synaptegrins) respond to the same diverse pharmacological agents that influence integrin attachment as well as LTP stabilization (Bahr et al., 1997), but they also can exhibit distinguishing biochemical characteristics (see Capaldi et al., 1997). Synaptegrins likely represent specialized adhesion molecules that, in light of their integrin-like nature, provide morphological constraint to "lock in" synaptic regulatory signaling.

RGDS-containing matrix proteins (e.g., fibronectin, vitronectin) are necessary for the adhesion response mediated by integrin-type receptors. Fibronectin mRNA and protein increased after intense neuronal stimulation (Hoffman et al., 1998c) perhaps to coordinate with the enhanced expression of RGDS-binding integrin 1 which also follows such synaptic activity (see Pinkstaff et al., 1998). This suggests that components of the synaptic basal lamina, the highly specialized matrix interposed between pre- and postsynaptic membranes, undergo use-dependent regulation to provide the appropriate contact substratum for network remodeling events. Other types of matrix components, e.g. a diverse array of proteoglycan species, also exhibit regulated expression in an activity-dependent manner (Lander et al., 1997; Lauri et al., 1999). Certain of these proteoglycans contribute to brain extracellular matrix and are produced by neurons (Lander et al., 1998), whereas fibronectin is induced predominately in glial cells (Hoffman et al., 1998c).
Neural cell adhesion molecules of the immunoglobulin superfamily and members of the cadherin family of adhesive receptors are structurally very different than integrins and related synaptegrins. However, NCAMs and cadherins are similar to integrin-type matrix receptors in that they are concentrated at synaptic zones (Persohn et al., 1989; Uchida et al., 1996; Tang et al., 1998; Huntley and Benson, 1999) and appear to play a role in plasticity mechanisms related to memory. For example, antibodies that interfere with interactions between NCAMs impaired learning in rats (Doyle et al., 1992a). Similar dissociation of NCAM complexes disrupted the early expression of hippocampal LTP (Lüthi et al., 1994; Rønn et al., 1995; Staubli et al., 1998; also see Muller et al., 1996) as did cadherin-cadherin blockers (Tang et al., 1998). Integrin antagonists, on the other hand, had no effect on the initial potentiation (Xiao et al., 1990; Bahr et al., 1997). These results indicate that distinct phases of synaptic plasticity involve different types of adhesive responses. Further evidence for this idea emerged by triggering LTP induction in hippocampal slices before the application of antibodies and peptides that inhibit cell adhesion.

Post-induction infusion of dissociating agents that target NCAMs or cadherins had no effect on previously established LTP (Lüthi et al., 1994; Staubli et al., 1998; Tang et al., 1998). This is not the case for integrin blockers that disrupt matrix recognition. RGDS peptides significantly blocked the stabilization of LTP when infused well before, immediately following, and 10 minutes after induction (Bahr et al., 1997). This led to the prediction that initial potentiation is vulnerable during the time latent adhesion sites are converted into active contacts for stabilizing membrane organization. As predicted, blockage of matrix contacts 25 minutes or more post-induction produced no detectable effect on the synaptic potentiation (Staubli et al., 1998). Together, these data constitute strong evidence that integrin-type matrix recognition and subsequent signaling are necessary during the critical consolidation period for LTP, a period with close correspondence to memory consolidation across many paradigms. In contrast, non-integrin sites of contact are involved in early plasticity processes initiated by the LTP triggering stimuli.

Early events of the LTP development phase likely involve disassembly of structural complexes that maintain membrane configuration. Changes in the synaptic architecture conceivably are produced when contact sites and associated cytoskeletal components are modified to temporarily release their organizational constraints. To this end, the calcium-dependent protease calpain cleaves components of the cytoskeleton in a transient manner following brief infusion of hippocampal slices with NMDA (Bahr et al., 1995; also see Vanderklish et al., 1995). This is of interest since brief stimulation of NMDA receptors is the triggering mechanism of LTP and, more importantly, the concomitant activation of calpain has been shown to be critical for stable potentiation (Vanderklish et al., 1995, 1996). Similar disassembly processes should result when calpain signaling events target other structural elements including NCAMs (Covault et al., 1991; Sheppard et al., 1991), cadherins (Covault et al., 1991), certain types of integrins including synaptegrin-1 (Potts et al., 1995; Du et al., 1995; Bahr et al., 1997; Capaldi et al., 1997), and cytoskeletal components that exhibit increased susceptibility during adhesion-mediated signal transduction (see Fox et al., 1993; Earnest et al., 1995; Inomata et al., 1996; Rock et al., 1997; Bahr, 1999). One matrix receptor, synaptegrin-2, is unique in that it is resistant to proteolysis perhaps to maintain overall structural integrity during synaptic reconfiguration (Capaldi et al., 1997). In contrast, NCAMs are particularly susceptible to proteases in the intracellular compartment as well as in the extrasynaptic space. Relevant to this discussion, extracellular cleavage of NCAM isoforms occurs when NMDA receptors are briefly activated to mimic LTP induction (Hoffman et al., 1998). One of the cleavage products is markedly similar to that formed by tissue plasminogen activator (tPA) (Hoffman et al., 1998), a serine protease implicated in multiple forms of hippocampal synaptic plasticity (Huang et al., 1996; Baranes et al., 1998). More compelling is the finding that an inhibitor of tPA correspondingly blocks the extracellular NCAM fragmentation and the formation of stable LTP (Hoffman et al., 1998b). Conversely, application of tPA enhances the amount of potentiation induced by a single tetanus (Baranes et al., 1998). These results could help explain the differential roles NCAMs and matrix receptors have in synaptic remodeling. For
instance, brief bursts of neuronal activity may induce secretion of proteases (e.g., Baranes et al., 1998) and matrix proteins (Hoffman et al., 1998c) in order to facilitate 1) morphological changes via the disassembly of non-integrin sites and 2) stabilization of new membrane configurations by assembling integrin-type focal contacts.

The susceptibility of NCAMs to activity-dependent modification suggests another possibility in which changes in the extracellular NCAM domain(s) result in the direct or indirect regulation of synaptic signaling. Support for this comes from a substantial body of evidence that NCAM isoforms in hippocampus undergo specific phases of sialylation and desialylation over the course of memory acquisition and consolidation (see Doyle et al., 1992b; O'Connell et al., 1997). In fact, both phases appear to have a functional influence on AMPA receptors, a subclass of glutamate-gated channels implicated as being responsible for the expression of LTP. Enzymatic removal of N-acetylneuraminic acid and other acidic sugars of the sialic acid family causes the alteration of AMPA receptor binding properties (Hoffman et al., 1997) and, correspondingly, the complete prevention of LTP induction (Muller et al., 1996). As an alternative to removing the polysaccharide component of NCAMs, a recent report demonstrated that polysialic acid itself has a direct effect on the channel kinetics of AMPA receptors (Suppiramaniam et al., submitted). These studies together with a previous series (Hall et al., 1992, 1996a; Hall and Bahr, 1994) indicate that AMPA receptor functionality can be influenced by components of the synaptic membrane including, in particular, cell-surface adhesion molecules and their polysaccharide moiety.

Just as removal of charged sugars from NCAMs disrupts LTP (Muller et al., 1996), it recently was determined that removal of charged glycosaminoglycans (GAGs) also blocks stable potentiation (Lauri et al., 1999). GAGs such as heparan sulfate and keratan sulfate are highly sulfated polysaccharide side chains that decorate proteoglycans found in synaptic zones. The large saccharide component of proteoglycans causes heterogeneous electrophoretic mobility as exemplified in Figure 1A by the keratan sulfate-containing species found only in the brain. Several proteoglycans have been characterized as synapse-specific for being, for example, an integral component of synaptic vesicles (cf. Bahr et al., 1992a) or exclusive to forebrain SPM fractions (see Fig. 1A). Of particular interest in the context of this review are the syndecans, a family of transmembrane heparan sulfate proteoglycans that interact with PDZ domains and co-localize with the actin cytoskeleton (Cohen et al., 1998; Hsueh et al., 1998). Members of the syndecan family are in many ways similar to integrin-related molecules in that they 1) participate in cell surface adhesion (Liu et al., 1998), 2) are localized to focal adhesions (Woods and Couchman, 1994), 3) have appropriate contacts to facilitate communication between intracellular signaling elements and the extracellular environment (reviewed in Carey, 1997; Rapraeger and Ott, 1998), 4) are involved in the structural organization of postsynaptic domains (Hsueh et al., 1998; Ethell and Yamaguchi, 1999), and 5) participate in the formation of stable LTP (Lauri et al., 1999). The latter two characteristics are of particular note with regard to the link between GAG removal and loss of LTP. The extracellular domain of syndecans possesses many heparan sulfate chains that appear to facilitate morphological changes in dendritic spines (Ethell and Yamaguchi, 1999). Removal of GAG chains would result in the loss of key interactions between the GAGs and heparin-binding molecules including growth factors, matrix components, and adhesion molecules (see review by Carey, 1997). The loss of LTP expression upon removal of GAGs suggests that GAG linkages are important to regulatory events in central synapses. If this is the case, competitive blockade of such GAG interactions also would have detrimental consequences on the expression of plasticity. Lauri et al. (1999) showed that the competitive action of heparin-type GAGs or a soluble form of GAG-containing syndecan in hippocampal slices indeed blocks the stabilization of LTP but not its early expression. Thus, heparin-sensitive GAG interactions in the extrasynaptic space are similar to integrin-related contacts with regard to their involvement in early vs. latter phases of synaptic potentiation.
FIG. 1. Synaptic proteoglycans and GAG interactions. Panel A: Rat forebrain homogenate (H), forebrain synaptosome fraction (SPM), spleen (SP), skin (SN), liver (L), skeletal muscle (M₁), lung (LG), adrenal gland (A), kidney (K), smooth muscle (M₂), heart (HT), and Torpedo electric organ synaptic vesicles (SV) in equal aliquots (80 g membrane protein/lane) were subjected to SDS-PAGE (3-17% gradient gel) and immunoblotted with antibodies to the GAG keratan sulfate. The proteoglycan species concentrated in synaptic membranes (PGSPM) exhibits the characteristic mobility of a core protein modified by the addition of many GAG side chains, but is distinct from other synaptic proteoglycans (e.g., PGSV). The PGSPM is sensitive to trifluoromethanesulfonic acid and keratanase, appears to be labeled by alcian blue and [³H]heparin overlay,
and is absent from subcellular fractions containing myelin or mitochondria (not shown). The positions of 48-180 kDa standards are marked. Panel B: Binding of [3H]heparin was assessed using a filtration assay and the means of 3 independent determinations each were subjected to Scatchard analysis. Linear regression determined that the B_{max} values for hippocampal homogenate and SPM fractions were 38 and 110 pmol [3H]heparin/mg protein, respectively, with similar K_{D}’s of 550 nM.

The effects of heparin on plasticity processes may be related to the GAG’s ability to influence the regulatory nature of AMPA receptors that contributes to LTP-type synaptic enhancement. Specifically, low concentrations of sulfated GAGs decrease the ligand affinity expressed by the receptors (Hall et al., 1996b) and prolong the open state of AMPA-activated channels (see Fig. 2). This further supports the notion that GAG interactions, through proteoglycans endogenous to the synaptic environment, are involved in receptor modulation. Interestingly, earlier work on synaptic GAG interactions found that heparin preferentially binds to synaptic membranes with submicromolar affinity (Fig. 1B) and interacts with AMPA receptors either directly or indirectly (Bahr et al., 1992b; Hall et al., 1996). To test if GAGs can directly promote receptor modulation, it was necessary to isolate functional AMPA receptors away from all other elements of the synaptic membrane. Accordingly, conventional chromatographies were used to obtain receptors of >50% purity which, when reconstituted in a pure lipid bilayer, exhibited the characteristic multiple channel states of AMPA receptors present in their native membrane (Bahr et al., 1992b; Vodyanoy et al., 1993). Their single channel current fluctuations also responded to heparin with increased open time (Hall et al., 1996b; Sinnarajah et al., 1999) as was the case with essentially pure, immunoprecipitated AMPA receptors (Suppiramaniam et al., 1999), thus indicating a direct GAG effect. Note that receptor purification and reconstitution allowed the critical analysis of a potential modulatory factor on single AMPA channels with little change in the native kinetic and conductance properties (Vodyanoy et al., 1993). The expected pharmacological channel blockage also occurred in the absence and presence of the GAG influence (see Fig. 2). In sum, the reconstituted system is extremely valuable for the identification of GAG-AMPA receptor interactions that instill regulation.
The fact that GAGs can act on the pure AMPA receptor indicates the receptor has a binding site(s) that, when occupied by certain charged polysaccharides, mediates functional regulation. This site may be related to an allosteric modulatory site that interacts with the new pharmacological agents Ampakines resulting in changes in the AMPA channel open state which resemble those produced by sulfated GAG interactions (Fig. 2). However, the effects on receptors in their native membrane are completely different when comparing the action of Ampakines vs. GAGs. Ampakines increase AMPA currents and promote LTP induction (Arai et al., 1994; Staubli et al., 1994) while heparin-type GAG has no effect on baseline synaptic responses and inhibits stable potentiation (Lauri et al., 1999). The discrepancy may be due to the dense array of charged polysaccharides endogenous to synapses, thus keeping free GAG from reaching its
Ampakines are about 100 times more potent when acting on purified receptors than on membrane-associated receptors (Suppiramaniam et al., 1999). Perhaps without the modulatory influences of endogenous polysaccharides the AMPA receptors are able to be more responsive to the allosteric action of Ampakines.

Similarities between the effects of GAGs and polysialic acid indicate that these types of oligosaccharides interact with the same modulatory site or two comparable sites present on AMPA receptors. Correspondingly, both the heparinase (Lauri et al., 1999) and desialylation (Muller et al., 1996) studies discussed earlier point to the existence of synaptic polysaccharides that, once stripped away, no longer link AMPA receptors to structural matrices through extracellular and transmembrane domains of their core protein. Alternatively, it is plausible that instead of acting through an allosteric site, synaptic oligosaccharides promote homophilic interactions between the receptors to generate clustered channel events and concomitant waveform changes. Clustered channel activity indeed is promoted by heparin but not by uncharged GAGs (Hall et al., 1996b). Charged monomeric constituents of GAGs are ineffective (Hall et al., 1996b; Sinnarajah et al., 1999) suggesting that some kind of cage structure consisting of large polysaccharides is assembled around the extracellular field of AMPA receptors during early LTP processes. With this idea, the allosteric modulation is mediated by the homophilic contacts among receptors grouped together by GAG interactions. There is, in fact, strong evidence that GAGs enhance signaling by promoting receptor multimerization (Rapraeger, 1995). In the case of Ampakines, perhaps the allosteric modulatory site for self-self interactions is strengthened when an Ampakine binds to the same site or in close proximity to the homophilic connection.

Linkages through proteoglycans may promote receptor associations with intracellular regulatory devices, e.g. kinases (see Barria et al., 1997), and/or facilitate receptor reorganization/clustering (Xie et al., 1997; Song et al., 1998) that results in enhanced efficiency for receiving presynaptic signals. These two possibilities would, thereby, translate changes in structural matrices into the stable maintenance of synaptic modulation. The concept regarding molecular schematization for the alignment of transmitter release and reception during LTP likely entails, at least in part, coordinated AMPA receptor patterns which express the evident changes in response kinetics (see Ambros-Ingerson and Lynch, 1993; Xie et al., 1997). Correspondingly, it is of interest that GAGs induce the cooperative activation of AMPA channels and prolong their functional bursting (Hall et al., 1996b; Sinnarajah et al., 1999). These findings propose that the GAG side chains of appropriately positioned proteoglycans help form functional aggregates of AMPA channels during the structural cascade associated with LTP. It should be noted here that in the neuromuscular junction there is evidence that synaptic proteoglycans can influence postsynaptic receptor clustering as well as the organization of the presynaptic membrane (see Megeath and Fallon, 1998; Tsen et al., 1995). An obvious advantage of the proposed hypothesis is that it explains the disruption of LTP following removal of proteoglycan-linked GAGs and when native interactions are competitively blocked by GAGs having no ties to intra- or extracellular signaling elements. Interestingly, the effects of unattached GAG on pure AMPA receptors (Sinnarajah et al., 1999) and on hippocampal LTP (Lauri et al., 1999) are both dependent on the presence of charged sulfate groups. As mentioned earlier, the general action of cell-surface proteoglycans depends on both specific extracellular interactions through their GAG chains and intracellular linkages between the core protein and cytoskeletal and signaling molecules. The reason for the competitive GAG effect assumes then that free GAGs are unable to influence signaling events in an environment where multiple polysaccharides are providing ample structural/functional constraints. Evidence for this comes from a report previously mentioned where heparin was shown to modulate only those receptors whose native membrane is completely dissociated by solubilization (Hall et al., 1996b).

There are perhaps many types of cell surface adhesion molecules that contribute to the structural
organization and architecture of central synapses. This review compared those adhesion molecules that have been studied with regard to their involvement in different phases of synaptic potentiation, e.g. early induction vs. stable maintenance. It is concluded that non-integrin type contacts (i.e., NCAMs and cadherins) perform to first establish the potentiated configuration of the synapse, while integrin and synaptegrin matrix receptors are necessary to make the new synaptic morphology permanent. More specifically, activated matrix receptors are thought to form the intracellular signaling complexes that maintain the stable modulation of AMPA channel properties. Interestingly, extracellular components also are involved in the stabilization of the potentiated synaptic structure. The latter consolidation phase appears to entail complicated interactions between the transmitter-gated channels and the extrasynaptic domains of what may be a variety mucoproteins and mucopolysaccharides. Heparan sulfate proteoglycans have received much of the attention in this regard due to their tight binding of heparin, a close relative of the heparan sulfate GAGs. The presence of such proteoglycans in the brain has long been known from studies of glycosidase deficiencies, i.e. mucopolysaccharidoses, which cause among other things the accumulation of heparan sulfate and neurological dysfunction (see Kint et al., 1973; Dawson, 1979). Under normal physiological conditions, however, proteoglycans and other polysaccharide-containing ingredients of the synaptic environment appear to facilitate the functional aggregation of AMPA channels, either through an allosteric site or by receptor multimerization. Thus, extracellular linkages may direct the molecular reorganization events needed in order that intracellular signaling complexes can be assembled in the proper configuration for uninterrupted receptor regulation.

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Rapid Communication

Integrin-Type Signaling Has a Distinct Influence on NMDA-Induced Cytoskeletal Disassembly

Ben A. Bahr*
Department of Pharmaceutical Sciences and the Neurosciences Program, University of Connecticut, Storrs, Connecticut

Adhesion responses triggered by integrin-class matrix receptors have been implicated in the synaptic reorganization events necessary for certain types of neuronal plasticity. Hippocampal slice cultures were used to test whether the related structural transformations elicited by NMDA receptor stimulation are regulated by integrin-type signals. Infusing the slices with NMDA for a short period induced the expected disassembly of the cytoskeletal network, measured with antibodies that selectively recognize spectrin cleavage sites targeted by the protease calpain. Marked levels of the 150-kDa breakdown product (BDP) were produced, whereas concentrations of the parent spectrin were not changed. Interestingly, the calpain cleavage events were attenuated by 60% when integrin-type signaling was disrupted with the antagonist Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP). This effect was RGDS-dependent, was largely evident in synapse-dense dendritic areas, particularly in subfield CA1, and was abolished when the NMDA exposure period was >5 min. These findings suggest that only those cytoskeletal alterations associated with brief synaptic activity are regulated by intact contact zones. AMPA-type glutamate receptors also were tested because, like spectrin, they are targets for calpain. Brief NMDA treatment caused a 15% loss of AMPA receptor GluR1 carboxytermini and this modification was augmented to 32% in the presence of GRGDSP. Thus, although blockage of matrix recognition signals decreased spectrin's susceptibility to disassembly, it increased the susceptibility of AMPA receptors to proteolysis. These data indicate that integrin-type signaling complexes are appropriately positioned to govern cytoskeletal reconfiguration while stabilizing the structural nature of AMPA receptors.

Key words: adhesion molecules; calpain; spectrin breakdown; synaptic plasticity

The structural organization that integrates the intracellular and extracellular matrices involves complex adhesion dynamics mediated by integrin-type cell surface receptors. In the nervous system, interactions between extracellular integrin domains and the Arg-Gly-Asp-Ser (RGDS) sequence within components of the interstitial matrix have been implicated in migration, synaptogenesis, survival, and regeneration (see Reichardt and Tomaselli, 1991; Hynes, 1992; Lefcort et al., 1992). Interestingly, the transmission of such adhesive signals to intracellular networks also may be necessary for the functional (e.g., long-term potentiation [LTP]) and related structural modification of central synapses (see review: Bahr et al., 1999). Although there is evidence for the former, little connection has been made between integrin-related chemistries and changes in synaptic morphology.

Antagonists that disrupt integrin-type signaling exhibit similar selectivity for preventing hippocampal LTP as for blocking matrix recognition (Staubli et al., 1990; Xiao et al., 1991; Bahr et al., 1997). The effects on LTP occur without any disruption of the initial degree of potentiation before the eventual collapse of the stabilization phase. Interestingly, the adhesion responses associated with LTP resemble typical integrin dynamics (van Willigen et al., 1996; Newton et al., 1997) in that they are prolonged perhaps to activate signaling complexes (Bahr et al., 1997; Staubli et al., 1998). Activated adhesion complexes can in turn recruit Fyn/Shc pathways and focal adhesion kinase (FAK), the latter of which is linked to signaling events that regulate the cytoskeleton as well as gene expression (Hynes, 1992; Juliano and Haskill, 1993; Giancotti and Ruoslahti, 1999). The relationship between integrin-type signaling and cytoskeletal reorganization may help explain the changes in postsynaptic morphology found in associ-
ation with specific types of synaptic plasticity including hippocampal LTP (Lee et al., 1980; Chang and Greenough, 1984; Desmond and Levy, 1983; Buchs and Muller, 1996; Geisman et al., 1996; Maletic-Savatic et al., 1999). One likely participant in LTP-related structural alterations is calpain, a calcium-activated cysteine protease. In certain cell types, integrin-matrix interactions induce both the translocation of calpain to focal contacts and its activation in response to elevated intracellular calcium (Beckerle et al., 1987; Fox et al., 1993). As a result, the activated protease links integrin chemistries to multiple signaling cascades by influencing such targets as pp60 

MATERIALS AND METHODS

Slices of hippocampus (400 μm) were prepared from 11–12 days postnatal rats and distributed on Millipore culture membranes. All incubation conditions were at 37°C, and cultures were supplied every 2–3 days with media composed of 50% basal medium Eagle, 25% Earle’s balanced salts, 25% horse serum, and supplements previously detailed (Bahr et al., 1995). Slices at culture day 12–18 were preincubated with or without 400 μM hexapeptide from Calbiochem for 60 min, then treated with 200 μM NMDA for 5–20 min in the presence of physiological ion concentrations. Rapid quenching was followed using ice-cold buffer containing chelators, then the slices were immediately fixed in paraformaldehyde or homogenized in groups of 4–6 slices in 0.32 M sucrose, 10 mM Tris (pH 7.4), 2 mM EDTA, 1 mM EGTA, 0.4 mM phenylmethylsulfonyl fluoride, 10 mg/l antipain, and 2 mg/l each of leupeptin, apro- tinin, and pepstatin. Slices fixed for 3 hr were cryoprotected and carefully removed from the insert, then 20 μm sections were prepared. Using the avidin-biotin-peroxidase technique, sections were stained with affinity-purified antibodies (anti-BDP) to latter residues of the amino-terminal spectrin fragment produced by calpain (Bahr et al., 1995). Threshold digitization of immunostained area utilized the BIOQUANT 98 image analysis system (R & M Biometrics, Inc.) linked to an Olympus AX70 microscope. Homogenized samples were assessed for protein content and equal protein aliquots were analyzed by immunoblot for BDP, spectrin, and GluR1 as previously described (Bahr et al., 1995, 1996). Bands were scanned at high resolution and measured for integrated density with the BIOQUANT software.

RESULTS

NMDA receptors were briefly activated in hippocampal slice cultures to initiate the chemistries associated with certain forms of synaptic plasticity. As previously shown, NMDA caused rapid cytoskeletal disassembly as indicated by the cleavage of spectrin between Tyr115 and Gly116 of its 11th repeat unit by calpain (Bahr et al., 1995). Antibodies to the calpain recognition sequence were used to measure the induction of the 150-kDa amino-terminal breakdown product (BDP; see blot lanes 1

![Fig. 1. Proteolytic effects associated with NMDA receptor activation.](image-url)
Integrins Govern Structural Plasticity

Fig. 2. Immunocytochemistry (a, b) and threshold imaging (c, d) of the NMDA-induced spectrin breakdown response. Cultured hippocampal slices were treated with NMDA for 5 min in the absence (a, c) or presence (b, d) of GRGDSP as in Figure 1. Anti-BDP staining in CA1 was especially evident in stratum radiatum (sr) including along dendrites (see arrows), but was sparse in the stratum pyramidale (sp). The image area in (d) is 64% less than that in (c). Scale bar = 25 μm.

and 2 in Fig. 1A), an induction that was completely suppressed by the NMDA receptor antagonist AP5 (not shown). An NMDA exposure period of 5 min was used to induce consistent disassembly events mediated by calpain without causing any measurable change in total spectrin (Fig. 1B). The latter result corresponds with the finding that a 5 min NMDA treatment causes cleavage of only one percent of the total spectrin in hippocampal slices (del Cerro et al., 1994). To test if the kind of adhesion responses implicated in synaptic modulation are involved in regulating NMDA-induced cytoskeletal changes, the GRGDSP matrix recognition blocker shown to disrupt stable LTP was used. At similar levels used in LTP experiments (Bahr et al., 1997), the integrin antagonist significantly reduced the extent of cytoskeletal disassembly measured after slices were exposed to NMDA. Specifically, the BDP response to NMDA receptor activation was attenuated 58% by GRGDSP (P < 0.0001) although no effect occurred to levels of parent spectrin (Fig. 1A and 1B). GRGESP, a control peptide that does not block matrix recognition, was ineffective at reducing the BDP response (Fig. 1A, lanes 4 and 5; also see Fig. 4).

There is convincing evidence that the cytoskeletal modification indicated by the stable spectrin fragments occurs in hippocampal synapses as part of functional reconfiguration (Vanderklish et al., 1995, 1996). Correspondingly, the NMDA-induced BDP response in the present study was largely confined to dendritic zones of the hippocampal slices. Particularly in field CA1, newly exposed carboxytermini of BDP species were localized to numerous puncta in stratum radiatum whereas only sparsely found in the pyramidal layer (Fig. 2). Threshold imaging shows that the BDP reduction from disrupting integrin-type signals almost exclusively involves the punctate staining in dendrites (compare Fig. 2c vs. 2d). These findings indicate that the GRGDSP effect is largely evident in synapse–dense areas as opposed to cell body layers, the latter of which would be more indicative of pathogenic influences on the cytoskeleton. In fact, the GRGDSP-mediated reduction in the BDP response was completely eliminated when the NMDA infusion lasted more than 5 min (Fig. 3); such extended exposure periods are known to induce persistent cytoskeletal deterioration as compared to momentary changes that are associated with brief NMDA treatments (see Bahr et al., 1995). These data support the idea that integrin-type matrix recognition controls the transient calpain signals initiated
by physiological bursts of synaptic activity and not the pathogenic activation of the protease.

Integrin-type adhesion complexes control calpain targeting in such a way as to make spectrin more susceptible to proteolysis, as supported by the inverse effect of the integrin antagonist. To test if this increased vulnerability is a general feature associated with adhesive signals, another calpain target important for synaptic mechanisms was evaluated. Accordingly, the GluR1 subunit of AMPA receptors was measured with antibodies that recognize its carboxyterminal target for calpain (Bahr et al., 1996; Bi et al., 1996). As shown in Figure 1C, immunostaining of GluR1 carboxytermini was reduced by 15% (P = 0.02) in hippocampal slice cultures treated with a 5-min NMDA exposure. This effect and more extensive losses occurring with more severe insults were blocked by the selective calpain inhibitor N-benzyloxycarbonyl-L-leucyl-L-phenylalanyl-COOH. The matrix recognition blocker GRGDSP, on the other hand, enhanced the NMDA-mediated release of GluR1 carboxy termini to 32% (P < 0.001), apparently doubling the effect produced by the 5-min NMDA treatment (Fig. 1C and the lower panel in Fig. 4). Thus, integrin-type signaling seems to cause hippocampal AMPA receptors to be less susceptible to proteolysis, opposite to that observed for the spectrin breakdown response to NMDA (Fig. 4). As was the case for the BDP antigen, peptides without the RGDS sequence had no effect on NMDA-induced changes in GluR1.

**DISCUSSION**

The above results indicate that calpain-mediated spectrin disassembly occurs in response to brief NMDA receptor stimulation, a triggering event for LTP, and is attenuated by blocking the RGDS recognition site of matrix receptors. These findings add to the substantial body of evidence that integrin-type signal transduction facilitates cytoskeletal reorganization. More importantly, they provide evidence that chemistries involved in the functional nature of synaptic modification are also part of the associated structural plasticity. This follows largely from the specific effects blockage of matrix recognition has on synaptic potentiation without affecting baseline physiology, the NMDA current, the burst responses that trigger LTP, or the initial facilitation of synaptic activity that follows the bursts (Bahr et al., 1997). Alterations in postsynaptic morphology have been long suspected to play an important role in the persistence of LTP, especially with recent studies implicating three of the four major classes of molecules that regulate membrane shape (cadherins, the neural cell adhesion molecule superfamily, and integrins) as part of the mechanistic phases of synaptic modulation (see Bahr et al., 1999). Interestingly, only integrin-type contacts have been shown to be necessary for the stabilization of plasticity events classified as LTP. Disrupting such contacts in the present study caused a decrease in the spectrin breakdown response expressed in synaptic fields subsequent to NMDA receptor activation, thus the contact zones must govern cytoskeletal modulations. Spectrin fragmentation of this type has been shown to be vital for stable LTP (Vanderklish et al., 1995, 1996).

Spectrin is only one of several cytoskeletal components to exhibit increased proteolytic susceptibility during integrin-
mediated signaling, thereby leading to the destabilization of structural constraints (Fox et al., 1985, 1993; Huttenlocher et al., 1997). Just as spectrin becomes less vulnerable to calpain when integrin-type signals are disrupted, the same is true for talin (Inomata et al., 1996); talin links integrin complexes to actin filaments that are involved in structural and functional aspects of synapses including those that allow synaptic potentiation (Allison et al., 1998; Kim and Lisman, 1999). Integrin-type contact zones, thus, can design a cytoskeletal configuration with modifiable structural integrity through the disassembling action of calpain that targets both spectrin and actin matrices. Structural disassembly is likely part of the LTP development phase where temporary changes in the synaptic architecture conceivably are produced to promote the orientation of a more functionally efficient environment. Correspondingly, calpain has been shown to cleave components of the cytoskeleton in a transient manner after brief NMDA infusion or delivery of LTP-inducing theta bursts (Bahr et al., 1995; Vanderklish et al., 1995). There also is recent evidence that brain spectrin as well as α-actinin 2, an actin binding protein, attach directly to NMDA receptors (Allison et al., 1998; Wechsler and Teichberg, 1998). These attachments may assist integrin signaling complexes in the efficient and rapid disassembly of the local cytoskeletal network during LTP development.

While spectrin is less targeted by calpain when matrix recognition sites are blocked, it is surprising that the GluR1 subunit of AMPA receptors becomes more accessible to the protease. It is possible, then, that focal adhesion complexes are organized in such a way as to promote cytoskeletal modulation while at the same time preventing the proteolytic damage of AMPA receptors. Integrin-type signals may protect the receptors from severe levels of carboxyterminal modification after activation of NMDA currents. More specifically, activated integrin receptors may contribute to the intracellular signals that not only establish a new synaptic configuration but also stabilize AMPA channel properties and their functional regulation.

Adhesion responses responsible for synaptic conformity likely are mediated by integrin-type matrix receptors found expressed in the adult brain and, in many instances, appropriately localized to synaptic contacts (Bahr and Lynch, 1992; Grooms et al., 1993; Einheber et al., 1996; Nishimura et al., 1998; Pinkstaff et al., 1998, 1999). In concordance with the physiology studies, matrix receptors purified from synaptic membranes adhere to matrix proteins via the RGD integrin target sequence and respond to the same diverse pharmacological agents that influence LTP stabilization (Bahr and Lynch, 1992; Bahr et al., 1997; Capaldi et al., 1997). Thus, the isolated receptors are strong candidates for contributing to the synaptic processes involved in morphological and functional regulation. They also may be involved in the link evident between synaptic remodeling events and intracellular signals that lead to increased mRNA levels for growth factors as well as integrin subunits (Isackson et al., 1991; Gall et al., 1997; Pinkstaff et al., 1998). Such regulation in gene expression is thought to be a major part of the cellular recovery mechanisms activated by pathogenic episodes. As shown in the present study, integrin antagonists do not affect the spectrin breakdown response to pathological levels of NMDA stimulation, although involvement of calpain during such levels of stimuli is well established in hippocampal slice cultures (Bahr, 1995). This brings up two possibilities that could be of importance. Physiological or subphysiological levels of synaptic activity and the resulting adhesion responses reinforce endogenous repair systems to promote recovery from injury. Excitotoxic levels, on the other hand, likely disrupt matrix receptor signaling pathways that induce growth/regeneration. The connection between synaptic remodeling and neuroprotective signaling was evident in a recent study showing that a drug that enhances the chemistries of synaptic plasticity also increases neuronal recovery from pathogenic insults (Bendiske et al., 1999). Neuroprotection can be elicited through many signaling pathways such as those associated with FAK, eventually conducing the activation of mitogen-activated protein kinase (MAPK) cascade and the regulation of transcription factors and growth factor receptors (see Giancotti and Ruoslahti, 1999). There are also alternative pathways by which cell survival can be linked to structural remodeling. For instance, Rac mediates growth stimulatory signals in a way that correlates with its ability to organize the cytoskeleton rather than with its ability to activate MAPK (Joneston et al., 1996; Lamarche et al., 1996; Huang and Ingber, 1999). Thus, integrin-type signals may evoke a variety of molecular rearrangements that not only facilitate functional regulation but also promote the brain's capacity for self-repair.

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DELAYED AND ISOFORM-SPECIFIC EFFECT OF NMDA EXPOSURE ON NEURAL CELL ADHESION MOLECULES IN HIPPOCAMPUS

Keith B. Hoffman¹, Ben A. Murray², Gary Lynch³, Subramani Munirathinam⁴, and Ben A. Bahr⁴*

¹Ancile Pharmaceuticals, 10555 Science Center Drive, San Diego, CA 92121
²Division of Biology 139-74, California Institute of Technology, Pasadena, CA 91125
³Department of Psychiatry and Human Behavior, University of California, Irvine, CA 92697
⁴Department of Pharmaceutical Sciences and the Neurosciences Program, University of Connecticut, Storrs, CT 06269-2092

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Correspondence to: Ben A. Bahr
Dept. of Pharmaceutical Sciences and the Neurosciences Program
University of Connecticut, Storrs, CT 06269-2092
Tel: 860-486-6043; Fax: 860-486-6858
E-mail: Bahr@uconn.edu
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ABSTRACT

Brief stimulation of NMDA receptors has been shown to generate proteolytic fragments from the extracellular domain of neural cell adhesion molecules (NCAMs). In the present study, hippocampal slice cultures were used to demonstrate that such brief stimulation is followed by a delayed increase in the 180-kDa isoform NCAM-180. The slices were exposed to NMDA for 30 s followed by rapid quenching with the antagonist AP5. Immunoassays of the experimental samples indicated that concentrations of NCAM-180 were elevated above matched controls 2-3 h after the NMDA exposure but not at earlier or later time points. This effect was isoform-specific as concentrations of the 140-kDa NCAM species were not found to increase. Interestingly, similar selectivity was evident with prolonged infusions of NMDA where, in contrast to the effect of brief stimulation, NCAM-180 content was reduced to 50% while levels of NCAM-140 were unchanged. Together with previous findings, the data indicate that the synaptic chemistries activated by NMDA differentially regulate NCAM-180 at the translation level and by localized activation of proteases.
INTRODUCTION

Cell surface adhesion molecules and extracellular matrix components have been implicated in the structural plasticity associated with long-term potentiation (LTP) (see Hoffman, 1998; Bahr et al., 1999). It is of interest that inhibitory agents targeting the extracellular interactions of NCAM also disrupt the formation of LTP (Lüthi et al., 1994; Rønn et al., 1995), and cell surface expression of polysialic acid on NCAMs appears to be a prerequisite for the functional and structural modulation (Becker et al., 1996; Muller et al., 1996; Theodosis et al., 1999). Moreover, there is a correspondence during memory encoding with regards to the polysialylation (Doyle et al., 1992; Murphy et al., 1996). Modifying synaptic integrity likely involves a sequence of mild disassembly then re-establishment of membrane configuration that is mediated by NCAM and other adhesion receptors (see Muller et al., 1996; Bahr et al., 1997; Bahr, 2000; Foley et al., 2000). Relevant to the first step of the sequence, recent studies have shown that brief periods of NMDA stimulation cause an immediate increase in large fragments derived from extracellular domains of NCAM (Hoffman et al., 1998a; also see Endo et al., 1999ab). Further evidence indicates that this effect is mediated by a serine protease(s), possibly released from the cell as part of the neuronal response to brief NMDA receptor activity. Correspondingly, another ligand of excitatory amino acid receptors (kainate) has been shown to cause selective and correlated increases in serine protease activity and NCAM fragmentation in hippocampal field CA3 (Endo et al., 1999b). Besides the role of extracellular proteolysis in synaptic disassembly, modification of the cytoplasmic region of NCAM also may be necessary as a prerequisite event. Previous studies have shown that NCAM is cleaved and released by calpain (Covault et al., 1991; Sheppard et al., 1991a), a cysteine protease found concentrated in spines and dendrites (Siman et al., 1985; Perlmutter et al., 1988). Calpain produces lasting
effects on synaptic structures following the stimulation of NMDA receptors (Bahr et al., 1995) or after short bursts of afferent activity which elicit LTP (Vanderklish et al., 1995).

While intracellular cleavage of NCAM coupled to extracellular fragmentation is consonant with a disassembly step, there is the obvious question of whether, and with what delay, the released adhesion receptors are replaced for the essential reassembly step. The experiments reported here tested the prediction that the NMDA stimulation paradigm shown to produce rapid NCAM modification also triggers production of replacement adhesion molecules.

**MATERIALS AND METHODS**

The polyclonal anti-NCAM antibodies were used in this study as described (Murray and Jensen, 1992). Standard methods were utilized for the preparation of cultured hippocampal slices from postnatal day 11-12 rats (Stoppini et al., 1991; Bahr et al., 1995). Transverse slices of 400 μm were placed on Millicell culture plate inserts in contact with culture media (CM) on the underside and humidified air with 5% CO₂ on the surface. CM consisted of 50% Basal Medium Eagle, 25% Earl’s balanced salt solution, 25% horse serum, and supplements to the following final concentrations: 40 mM glucose, 0.5 mM ascorbic acid, 136 mM NaCl, 2.5 mM MgSO₄, 2 mM CaCl₂, 5 mM NaHCO₃, 3 mM glutamine, 20 mM HEPES buffer (pH 7.3 at 23°C), 1 mg/l insulin (Sigma, 24 I.U./mg), 5 units/ml penicillin, and 5 mg/l streptomycin. The slices were maintained at 37°C for 2-3 weeks with media changed on alternate days.

Brief NMDA stimulation was conducted as previously described for acute slices (Thibault et al., 1989). The slice cultures were pretreated with CM plus 850 μM spermine at 37°C for 15 min to facilitate NMDA receptor stimulation; for control slices, 100 μM AP5 was included at this step. The spermine solution was then removed and NMDA receptors were immediately
activated for 30 s with CM plus 400 μM NMDA, 2 mM extra CaCl₂ (4 mM total), and 1 mM glycine. An NMDA-free solution was used in the case of control slices with the addition of 400 μM AP5. Following the 30 s incubation, the solutions were aspirated and CM plus 2 mM AP5 (high concentration to rapidly quench a majority of NMDA receptors) was added to all slices for 5 min. The slices subsequently were washed three times in CM plus 50 μM AP5, after which CM alone was added to the tissue wells for all remaining incubation times. For extended stimulation, a set of slices were treated with CM plus 150 μM NMDA and 4 mM total CaCl₂ at 37°C for 2 h; the controls were treated similarly but with the addition of 400 μM AP5. Care was taken to maintain all solutions at 37°C.

Culture wells were aspirated at the appropriate time and individual inserts were flooded with ice-cold harvest buffer containing protease inhibitors (10 mg/L antipain and 2 mg/L each of aprotinin, leupeptin, and pepstatin A). The slices were gently removed with a brush and washed by centrifugation. Samples of 5-8 slices each were then subjected to one freeze-thaw cycle and sonicated in hyposmotic lysis buffer (8 mM HEPES, pH 7.4, 0.3 mM EGTA, 1 mM EDTA, and the protease inhibitors listed above). Equal protein amounts of the sonicated samples (75 μg) were denatured in 3% (vol/vol) 2-mercaptoethanol and 2.5% (wt/vol) sodium dodecyl sulfate (SDS) at 100°C for 3 min. The samples were subjected to gel electrophoresis on 3-17% polyacrylamide gradient gels. Proteins then were transferred to a nitrocellulose support (0.2-μm pore size) with the Bio-Rad transblot system for 5-10 h and stained with anti-NCAM antibodies (3-6 μg/ml). Color development of the immunoblots was terminated before maximal intensity was reached in order to ensure a linear relationship with increasing sample load. Routine linearity tests have been conducted and the typical protein vs. immunoreactivity correlation for the colorimetric assay (r = 0.99) was greater than that for the chemiluminescence method (r = 0.96) while having similar sensitivities. A computerized image analysis system was used to
RESULTS

Initial experiments were conducted to confirm that NCAM species in cultured hippocampal slices possess the characteristics of those found in the adult brain. Immunoblot samples from slices harvested immediately after preparation (culture day 0, i.e., CD 0) displayed a large portion of diffuse immunostaining located above the 140- and 180-kDa bands which is consistent with the heavy glycosylation of immature NCAM isoforms (Fig. 1). Over 10 days in culture the smeared immunoreactivity decreased dramatically, closely matching the developmental change in NCAM glycosylation that occurs in vivo (Choung and Edelman, 1984; Regan, 1991; Seki and Arai, 1993). In slices at CD ≥10, the NCAM species of 120, 140 and 180 kDa were indistinguishable with regard to glycosylation state and isoform ratios from those antigens in hippocampal homogenates and synaptosomes freshly prepared from adult rats (Fig. 1). The isoforms remained at stable levels in the slices between CD 10-60 as previously indicated (Bahr et al., 1995). In addition to the usual three isoforms, lower molecular weight bands also were recognized by anti-NCAM. These antigens of 60-75 kDa most likely represent NCAM cleavage products (Hoffman et al., 1982; Cunningham et al., 1983; Hoffman et al., 1998a). Surprisingly, concentrations of the 65- and 75-kDa species stabilized between CD 5-30, which would be consistent with being byproducts of parent isoforms during normal turnover events.

To examine the NCAM response to brief NMDA stimulation, slices at CD 15-20 were exposed to the agonist using a fast activation-quenching sequence. A high concentration of
NMDA was used (400 μM) in order to allow diffusion of the agonist into the tissue and maximum receptor stimulation in the 30-s period before antagonist was applied. This paradigm has been shown to produce a long-lasting increase in hippocampal responses (Thibault et al., 1989; Malenka, 1991) as well as NCAM fragmentation (Hoffman et al., 1998a). Generation of replacement adhesion molecules was assessed 0.2-18 h after the burst of NMDA receptor activity. As shown in Fig. 2A, slices harvested 2-3 h post-stimulation contain more NCAM-180 than do control slices in which NMDA was applied in the presence of the antagonist AP5. On the other hand, changes in the staining of NCAM-140 and NCAM-120 were not apparent. Experimental vs. matched control comparisons confirmed that the increase in NCAM-180 is statistically significant 2-3 h after the NMDA exposure (p=0.001, n=23; two-tailed, paired t-test), but not so when measured at 0.2, 5-6, or 10-18 h post-stimulation (Fig. 2B). Additional slices were harvested 2-3 days after the brief NMDA treatment to test for delayed pathogenesis, but little change in NCAM-180 levels was found (-5±13%; n=9).

The alteration in expression of NCAM-180 observed 2-3 h after neuronal stimulation was determined to be isoform-specific. Within-sample comparison was used to plot the AP5-sensitive change in NCAM-180 vs. that regarding NCAM-140. As evident in Figure 3, the NMDA effect on NCAM-180 almost exclusively translated as a positive modification while NCAM-140 levels exhibited variable shifts in both the positive and negative direction. Furthermore, the changes in the two isoforms were not correlated (r=0.21), indicating that negligible NCAM-140 up-regulation occurs correspondingly in synapses where NCAM-180 expression is enhanced. Similar analysis of the 120-kDa variant showed no consistent effect (not shown), nor was there any induced regulation of either NCAM-120 or NCAM-140 in slices harvested >5 h after NMDA exposure.
The above results indicate selective up-regulation of an NCAM isoform in response to brief activation of NMDA receptors. Further experiments addressed whether intra- and/or extracellular NCAM modification exhibits similar isoform selectivity. Although NCAM fragmentation has been observed after brief stimulation, extended NMDA treatment was needed to produce measurable changes in the immunoreactivity of the parent isoforms. Surprisingly, the same 180-kDa isoform found to be up-regulated after 30 s of NMDA stimulation also was selectively reduced by ~50% in slices subjected to prolonged NMDA perfusions of 1-2 h (Fig. 4). This reduction was AP5-sensitive and isoform-specific as no such decrease was exhibited by NCAM-140. NCAM-120 was not consistently stained to allow quantitation.

DISCUSSION

The described results establish that brief activation of NMDA receptors elicits a delayed and transient up-regulation of one particular NCAM variant. The finding may have implications concerning plasticity mechanisms since it corresponds to that expected from LTP studies where intense afferent stimulation was used (Fazeli et al., 1994; Hoffman et al., 1998b). The increased expression was specific for NCAM-180, the same isoform found to be selectively reduced when NMDA stimulation was sufficiently prolonged to cause a measurable decrease. The reduction is possibly related to the coupled release and degradation reported previously. The isoform specificity in the present study corresponds with the selective targeting exhibited by the intracellular protease calpain (see Covault et al., 1991). Note, however, that other studies showed no such isoform selectivity upon induction of calpain (Sheppard et al., 1991ab) or extracellular fragmentation (Hoffman et al., 1998a; Endo et al., 1999b). Perhaps NMDA channel functions under certain conditions produce targeted effects through interactions with precise
structural networks in central synapses (e.g., see Allison et al., 1998; Wechsler and Teichberg, 1998). The exact contribution of intra- and extracellular proteases in the selective reduction of NCAM-180 will require an array of careful pharmacological studies.

The up-regulation of the 180-kDa isoform perhaps is indicative of a feedback system that governs mRNA splicing in response to intra- and/or extracellular modification. It is of interest in this regard that such regulation of mRNA splicing may affect NCAM function and membrane morphology (Murray et al., 1986) to facilitate the mechanochemistries underlying synaptic potentiation. Studies have clearly shown that differential expression of cell adhesion molecule genes is under strict control (see Edelman and Jones, 1998). Transcriptional regulation of the NCAM gene, however, is an unlikely scenario in the present context for such regulation would augment alternatively spliced transcripts encoding multiple isoforms (Cunningham et al., 1987; Santoni et al., 1987; Walsh, 1988). Another possible explanation for the increased NCAM-180 immunoreactivity may be as a result of activated enzymes controlling polysialylation, an NCAM modification also implicated in synaptic plasticity (Doyle et al., 1992; Murphy et al., 1996). There are two reasons to discount this possibility. First, both the 140- and 180-kDa isoforms are subjected to similar regulatory events controlling their polysialylation over several days in cultured hippocampal slices, and there is no reason to assume that enzymes that attach polysialic acid residues to NCAM-180 do not act equally on NCAM-140. Second, the delayed nature of the up-regulation agrees better with a change in protein synthesis than with rapid enzymatic induction triggered by NMDA.

Whether elevated expression is coupled to partial digestion of the NCAM population during plasticity processes is not certain. First steps have been made in gaining insight in this matter by using selective protease inhibitors that block the NCAM cleavage effect without disrupting other signs of NMDA receptor activation (Hoffman et al., 1998b, 2000). The
resulting suppression of the NMDA-driven breakdown fragments was accompanied by a reduced capacity for stable LTP and kindling. Understanding potential links between the delayed response by NCAM-180 expression and the stabilization of physiological changes is difficult considering the mechanistic phases of synaptic modulation. In activated synapses, the initial release of NCAM likely promotes disassembly of the membrane architecture, a process also complicated by the involvement of another class of adhesive sites, i.e., the integrin family (Bahr, 2000). Interestingly, the digestion of NCAM could be more than a component or consequence of the disassembly process. Besides the potential role in destabilizing synaptic integrity, NCAM fragmentation may remove an enzymatic influence from the synapse or, alternatively, release a soluble NCAM species to modify extracellular targets (see Skladchikova et al., 1999). One or both of these possibilities could be necessary for reconfiguration of the synaptic environment.

After the disassembly phase, stabilization of plasticity has been found to require the adhesion response of the integrin class of adhesion molecules but not that of NCAM (Lüthi et al., 1994; Bahr et al., 1997). Thus, NCAM-mediated contacts are especially needed early after LTP initiation is triggered, perhaps to orchestrate selective disassembly. Although the NCAM sites are not part of the latter stages of LTP induction, NCAM regulation may be important for the very late consolidation. Corresponding to the temporal properties of the NCAM-180 increase described, several other laboratories have identified similarly delayed and transient induction of protein synthesis that is essential for the late consolidation phase for permanent plasticity (see Krug et al., 1984; Angenstein et al., 1992; Fazeli et al., 1993; Huang and Kandel, 1994; Nayak et al., 1998). Thus, vital proteins besides NCAM-180 are believed to be up-regulated for long-term maintenance of synaptic potentiation including matrix proteins (Hoffman et al., 1998c) and AMPA receptors (Nayak et al., 1998). Together with the present results, this would imply that
failure to reconstruct NCAM contacts and important structural organization within a critical sequence of adhesion and matrix responses could result in a reversal of synaptic remodeling.

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FIGURE LEGENDS

FIG. 1.

Maturation of NCAM isoforms in organotypic cultures of hippocampus. Postnatal day 11 slices (6-8 per sample) were harvested over 0-30 culture days (CD) and equal protein aliquots (40 µg) were processed for immunoblotting with anti-NCAM antibodies; control slices (CD 0) were frozen immediately after preparation and stored at -70°C for 30 days before processing. For comparison, fresh hippocampal samples (40 µg protein each) of synaptosomal plasma membranes (S) and tissue homogenate (H) from a 3-month-old rat were included on the same blot. The electrophoretic positions of the 180-, 140- and 120-kDa NCAM isoforms are shown.
FIG. 2.

Effects of brief NMDA treatment on the immunolabeling of NCAM isoforms. (A) Hippocampal slice cultures pretreated without (-; experimental) with (+; control) AP5 were subjected to a 30-sec NMDA exposure, followed by rapid quenching with antagonist (AP5 was continuously present in controls). Groups of 6 slices were harvested at 2 h post-stimulation and equal protein aliquots (75 µg) were processed for anti-NCAM immunoblotting; the migration pattern for the three isoforms is shown. (B) Paired samples of experimental and control slices were treated with NMDA and processed for immunoblotting 0.2 h (n=8 pairs), 2-3 h (n=23), 5-6 h (n=8), and 10-18 h (n=6) post-stimulation. Image analysis was used to determine the change in NCAM-180 immunoreactivity in experimental samples as compared to control samples; the mean change (± SEM) among paired samples is shown for each post-stimulation time. The effect at 2-3 h post-treatment is greater than that of the three other time points analyzed (p=0.015; two-tailed, one-sample t-test adjusted for a four-group study).
FIG. 3.

Scatter plot of the NMDA-induced change in NCAM-180 vs. that in NCAM-140 at 2-3 h post-stimulation. Each datum represents the percent change in immunoreactivity for each isoform in individual experimental samples as compared to paired controls (see Fig. 2B). While the NCAM-140 data are randomly distributed about the dotted zero line, the NCAM-180 data predominately exhibit increased levels.
FIG. 4.

Effects of extended NMDA treatment on the immunolabeling of the 180- and 140-kDa NCAM isoforms. Hippocampal slices were exposed to 150 μM NMDA for 2 h in the presence (immunoblot lane 1) or absence of AP5 (lane 2). Paired samples were processed for immunoblotting and image analysis in order to determine the AP5-sensitive change in the immunoreactivity of NCAM-180 (n=8 pairs) and NCAM-140 (n=8); the mean change ± SEM is shown for each isoform. *Two-tailed, one-sample t-test: p<0.02.