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Influencing Antibody-Mediated Attenuation of Methamphetamine CNS Distribution through Vaccine Linker Design

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Abstract

Active vaccination examining a single hapten engendered with a series of peptidic linkers has resulted in the production of antimethamphetamine antibodies. Given the limited chemical complexity of methamphetamine, the structure of the linker species embedded within the hapten could have a substantial effect on the ultimate efficacy of the resulting vaccines. Herein, we investigate linker effects by generating a series of methamphetamine haptens that harbor a linker with varying amino acid identity, peptide length, and associated carrier protein. Independent changes in each of these parameters were found to result in alterations in both the quantity and quality of the antibodies induced by vaccination. Although it was found that the consequence of the linker design was also dependent on the identity of the carrier protein, we demonstrate overall that the inclusion of a short, structurally simple, amino acid linker benefits the efficacy of a methamphetamine vaccine in limiting brain penetration of the free drug.

Graphical Abstract

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Author Contributions

Hapten synthesis and conjugation were performed by M.G., P.O.M., and A.D.; in vitro (ELISA and RIA) analyses were performed by M.G.; vaccination and blood/brain distribution studies were performed by C.J.W. K.D.J. oversaw the direction of the study. All authors have given approval to the final version of the manuscript. M.G., P.O.M., and C.J.W. contributed equally to this project.

Notes

The authors declare no competing financial interest.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscchemneuro.6b00389. Conjugation efficiency; experimental procedures on hapten synthesis and characterization, KLH-, TT-conjugation, active immunization, titer determination by ELISA, RIA analysis, and analysis of MA levels in serum and brain (PDF)

Keywords

Methamphetamine; vaccine; linker design; hapten; CNS distribution; antimethamphetamine antibody

In recent years, the abuse of synthetic psychoactive drugs (SPDs) has imposed a substantial health burden on society.^{1–4} Amphetamine-type stimulants (ATS) comprise a subset of SPDs whose members include amphetamine and methamphetamine (MA), among others. The abuse of ATS is a global and growing phenomenon—there has been a pronounced increase in their production and export during the past decade.^{5,6} The effects of MA and other ATS on the central nervous system (CNS) include euphoria, intensified emotions, altered self-esteem, increased alertness, aggression and sexual appetite.^{7–10} Furthermore, the abuse of MA has led to increased rates of presentation for cardiovascular and cerebrovascular pathologies.^{11,12} MA use can also quickly lead to addiction, increasing the risk of overdose, which is a significant source of mortality across multiple populations.^{13–16} Treatments for MA addiction are somewhat limited and are mainly based on behavioral therapies.¹⁷ However, the severity of MA withdrawal symptoms negatively impacts the ability of MA-dependent patients to maintain abstinence; most MA addicts relapse within 3 years of seeking treatment.^{18,19} Considering the widespread and rampant abuse of ATS, it is clear that improved measures to abate their effects and help patients to achieve and sustain abstinence are needed. Evolving an effective vaccine against MA would constitute a major breakthrough in the area, since the antibodies (Abs) induced by vaccination would capture the drug before it can cross the brain barrier, preventing its psychoactive and reinforcing effects from taking hold.

MA by itself cannot elicit activation of the Ab-generating B cells and helper T cells; hence, its presentation to the immune system must be initiated through a conjugate vaccine, which chemically links a homologue of the abused drug to an immunogenic protein such as tetanus toxoid (TT) or keyhole limpet hemocyanin (KLH).^{20–22} Crucially, such vaccines should elicit high concentrations of high affinity MA-specific Abs in order to be maximally effective. The challenge posed by developing a reproducibly robust antibody response across all members of a population while in pursuit of this goal has resulted in a shift toward direct infusion of monoclonal anti-MA Abs (passive vaccination), rather than promoting *in situ* production (active vaccination).^{23–26} However, because the latter offers advantages (in terms of prospective protection, ease of administration, and cost), further efforts to improve hapten design are warranted.

We have previously shown that incorporation of a diglycine linker into a nicotine hapten significantly enhanced both concentration and affinity of the resulting Abs.²⁷ Pravetoni et al. have also demonstrated an increase in Ab titers against oxycodone when glycine residues were introduced into the linker for their hapten.^{28,29} Inspired by these observations, we designed hapten **1**-GG to include a diglycine linker between the 4-carbon spacer group distal to the MA core and a glutarate handle, which can then be covalently attached to the carrier protein (Figure 1). In order to clarify if the peptide linker identity has an influence on MA-affinity and binding capacity of the Abs induced by vaccination, we also utilized the other small, hydrophobic amino acids alanine and proline to prepare dipeptides **1**-PG (Pro-Gly), **1**-PA (Pro-Ala), and **1**-A₂ (Ala-Ala).³⁰ An MA-hapten with no amino acid linker, **1**-0, was included as an additional structural control.

RESULTS AND DISCUSSION

Haptens of type **1** were synthesized using straightforward coupling chemistry. Incorporation of the peptidic linkers was accomplished using a mild *N*-hydroxysuccinimide (NHS) ester cross-linking strategy, in order to suppress racemization of the stereogenic carbon in the amphetamine moiety. Coupling of **3** (prepared from **2** as previously described)³¹ with NHS-activated esters of Boc-dipeptides followed by Boc cleavage gave intermediates **4a–d** (Scheme 1). Subsequent introduction of monomethyl glutarate and saponification to release the acid gave haptens **1**-GG (Gly-Gly), **1**-PG (Pro-Gly), **1**-PA (Pro-Ala), and **1**-A₂ (Ala-Ala). Hapten **1**-0 was prepared in a similar fashion via direct coupling of **3** with NHS-activated monomethyl glutarate followed by basic hydrolysis. Utilizing the same strategy, we also synthesized **1**-A₁ and **1**-A₃, which harbor mono- and trialanine linkers, respectively (Scheme 1), to probe on the effect of the alanine residue in eliciting Ab response.

Haptens of type **1** were then activated by reaction with EDC/NHS and conjugated to KLH as the immunogenic carrier protein. The resulting KLH-conjugates were formulated using a combination of alum and phosphorothionated CpG ODN 1826, as this vaccine formulation has proven effective in enhancing immune response in rodents and humans.³² Next, the efficacies of the five KLH conjugate vaccines were determined by vaccination of Swiss Webster mice (~170 µg KLH-conjugate/mouse). An initial tail bleed was performed on day 28 followed by a cardiac bleed at 42 days post vaccination. Mouse sera were then analyzed for Ab titers by ELISA, using a BSA-linked MA immunogen as the coating antigen (Figure 2A).³³ To determine the concentrations and MA-affinities of the resulting Abs, we conducted a competitive radioimmunoassay (RIA) using tritiated MA as the tracer.³¹

Several intriguing observations can be inferred from the results of these experiments (Figure 2B–D). While Ab titers induced by **1**-0-KLH remained high over the course of the vaccination schedule, those of the dipeptide-KLH conjugates were notably lower overall, with a significant decrease in titers observed between days 28 and 42 for **1**-GG, **1**-PG, and **1**-PA (Figure 2B). In contrast, we observed a boost in ELISA titers for the hapten harboring the Ala-Ala dipeptide (**1**-A₂). Because Ab titers are partly dependent on the coating antigen used in ELISA, we next determined the MA-affinities and concentrations of the elicited Abs to allow for unbiased comparison of the MA haptens. In general, all Abs raised against the dipeptide-KLH conjugates displayed greater MA-affinity compared to those raised against

1-0 (Figure 2C), signifying that the dipeptide linker enhances the binding affinity of the elicited Abs. Ab concentrations correlated well with the ELISA titers but varied inversely as MA affinity (Figure 2B–D). Although the dipeptide-KLH conjugates elicited antibodies with tighter binding affinities toward MA, the resultant Abs concentrations were comparably lower than that induced by **1-0-KLH** (Figure 2D). For example, **1-PG-KLH** elicited Abs with the greatest MA-affinity (with a K_d value of $0.56\ \mu\text{M}$, which is ~3-fold lower than that of **1-0**) but at significantly lower concentrations and ELISA titers.

Among the four dipeptide-KLH conjugates, **1-A₂-KLH** induced the highest ELISA titers and Ab concentrations with modest MA-affinity. We further probed the effect of the alanine residue by synthesizing monoalanine (**1-A₁**) and trialanine (**1-A₃**) variants (Scheme 1). Both **1-A₁-KLH** and **1-A₃-KLH** elicited Abs that had comparable K_d 's and concentrations as the dialanine counterpart (Figure 2B,C). ELISA analysis, however, showed remarkably high titers that are comparable to those of **1-0-KLH** on day 42 (Figure 2B).

To complement our linker assessment studies, we also looked at altering the carrier protein to examine potential carrier protein-T cell epitope effects; thus, the carrier protein TT was engaged. In contrast to KLH, TT has been successfully used in human vaccination for years without untoward side effects. Moreover, previous studies have shown that TT affords high hapten densities, which has translated to robust vaccine response.^{34–36} Haptens **1-0**, **1-A₁** and **1-A₂** were conjugated to TT via the EDC/NHS cross-linking strategy, resulting in comparable conjugation efficiencies as determined by MALDI-TOF analysis (Figure S1). The resulting TT-conjugates were also formulated with alum/CpG ODN 1826 prior to dosing. We note that in the subsequent studies, we immunized mice with a lower dose of the TT-conjugates, about half the amount we had used for the KLH conjugates.

As shown in Figure 3A, the TT-conjugates elicited Abs with exceptionally high ELISA titers on day 28. By the end of the study, the titers for **1-0-TT**, **1-A₁-TT** and **1-A₂-TT** reverted to a level similar to those observed with the KLH-conjugates (Figures 2B and 3A). In addition, we note that the **1-A₁-TT** conjugate evoked Abs with poorer affinity toward MA, as compared to the KLH-linked version, while those Abs induced by **1-0** and **1-A₂** demonstrated similar MA affinities when coupled to either carrier protein (Figures 2C and 3B). Interestingly, the Ab concentration induced by **1-A₁-TT** was significantly higher, consistent with the titer results obtained by ELISA (Figure 3A,C).

In order to assess functional efficacy, we studied the ability of the Abs induced by each TT-conjugate to sequester MA in the serum and limit brain penetration. Immunization with the TT-conjugates proved to be effective in reducing the brain penetration of MA as depicted in Figure 4. Among the three groups, the **1-A₁-TT**-vaccinated mice sequestered greater amounts of MA in serum (Figure 4A) resulting in a $\text{brain}_{[\text{MA}]}/\text{serum}_{[\text{MA}]}$ ratio of 1.6, which was 15-fold lower than that observed in the naive cohort (Figure 4C). On the other hand, the $\text{brain}_{[\text{MA}]}/\text{serum}_{[\text{MA}]}$ ratios in **1-0-TT**- and **1-A₂-TT**-vaccinated mice were comparable at 3.6 and 3.4, respectively, but were still notably better at sequestering MA than the nonvaccinated mice (Figure 4C).

Overall, Ab affinity and concentration are the two main factors that need to be taken into account for active vaccination strategies, and these may vary with (a) vaccine dose (b) carrier protein, or (c) hapten design (including linker complexity), which has been our target in this study. Our results show that incorporation of a dipeptide linker (as in **1**-GG, **1**-PG, **1**-PA, **1**-A₂) generally led to the induction of Abs with increased affinity toward MA, although these Abs were produced at lower concentrations relative to those induced by **1**-0. Among these four dipeptide-KLH conjugates, **1**-A₂-KLH evoked Abs with the highest concentration and titers. Therefore, we next investigated the impact of linker length on Ab MA metrics in the context of alanine-containing linkers. Interestingly, the mono- and trialanine counterparts produced Abs with comparable MA-affinities and concentrations to **1**-A₂-KLH, although both examples did demonstrate remarkably higher titers than their dipeptide comparator. These differences may arise from altered antigen recognition, as even single amino acid changes are sufficient to alter T-cell affinity.³⁷

We have also demonstrated that utilizing TT as the carrier protein generates Abs with high and early peaking ELISA titers. Intriguingly, despite the relatively modest MA affinities, the Abs induced by the TT-conjugates were efficacious in limiting the CNS penetration of MA, with those of **1**-A₁-TT being the most effective among the three groups. Combined with the **1**-A₁-KLH data, these results indicate that incorporation of a single amino acid species into the linker region can improve the functional response of a MA hapten. Given the current barriers to clinical adoption of an active vaccination strategy against MA and other ATS, investigations into the general application of this strategy across several hapten structures are warranted.

In pursuit of a general method, it should be noted that the variable impact on antibody affinity for **1**-A₁ when conjugated to TT versus KLH does indicate that linker optimization is not fully independent from the selection of carrier protein, likely due to the different T-cell epitopes that these carriers present to the immune system. Additionally, variability in response has previously been observed due to haptenization ratios on TT, although this pattern was not seen for the vaccines studied here, potentially due to differences in MHC display between the groups.³⁴ Nevertheless, this demonstrated use of amino acid containing linkers to variously alter the level of production and affinity of Abs indicates the possibility of “tuning” vaccine responses against small molecules. Indeed, by accounting for the relative affinities of a small molecule for its target and for the vaccine-induced Ab, it is plausible to envision this method being used to produce Abs that can achieve disparate pharmacokinetic outcomes depending on the desired application. Further studies on this topic are currently ongoing and will be published in due course.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

Ab	antibody
ATS	amphetamine-type stimulant
CNS	central nervous system
EDC	1-ethyl-3-(3-(dimethylamino)propyl)-carbodiimide
ELISA	enzyme-linked immunosorbent assay
KLH	keyhole limpet hemocyanin
MA	methamphetamine
MHC	major histocompatibility complex
NHS	<i>N</i> -hydroxysuccinimide
SPD	synthetic psychoactive drug
TT	tetanus toxoid

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Figure 1.
Structures of MA haptens with varying dipeptide linkers.

Figure 2.

Anti-MA antibody (Ab) titers, affinities, and antibody concentrations from **1**-KLH vaccinated mice ($n = 4$) with alum/CpG ODN 1826 as adjuvant. (A) BSA-linked MA immunogen used as the coating antigen in ELISA; (B) midpoint titers as determined by ELISA [one-way ANOVA: $P < 0.001$ (day 28), $P = 0.011$ (day 42)]; (C) dissociation constants (K_d); and (D) Ab concentrations (one-way ANOVA: $P = 0.003$) as determined by competitive RIA using pooled sera (on day 42). RIA data were obtained in duplicate. Errors represent SEM.

Figure 3.

Anti-MA antibody (Ab) titers, affinities, antibody concentrations from 1-0, 1-A₁₋₂-TT vaccinated mice ($n = 6$ on day 28, $n = 4$ on day 48) with alum/CpG ODN 1826 as adjuvant. (A) Midpoint titers as determined by ELISA [one-way ANOVA: $P = 0.024$ (day 28), $P = 0.005$ (day 48)]; (B) dissociation constants (K_d , one-way ANOVA: $P = 0.003$); and (C) Ab concentrations (one-way ANOVA: $P < 0.001$) as determined by competitive RIA using pooled sera (on day 48). RIA data were obtained in duplicate. Errors represent SEM.

Figure 4.

Serum and brain levels of MA. Data shown as (A) serum concentrations (one-way ANOVA: $P = 0.022$), (B) brain concentrations, and (C) brain to serum concentration ratio of MA at $t = 15$ min following a single intraperitoneal dose of MA (at 2 mg/kg body weight) to 1-0, 1-A₁₋₂-TT vaccinated or naive mice ($n = 2$). One-way ANOVA: $P = 0.047$.

Scheme 1. Preparation of MA Haptens^a

^aReactions and conditions: (a) TEA then Boc-dipeptide-OSu in DMF, 0.5 h at rt; (b) 4 N HCl in dioxane, 1 h at rt; (c) TEA then monomethyl glutarate-OSu in DMF, 0.5 h at rt; (d) 1 N LiOH in MeOH, 1 h at rt; (e) TEA then Boc-Ala-OSu in DMF, 0.5 h at rt; (f) TEA then Boc-Ala-Ala-OSu in DMF, 0.5 h at rt.