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Studies on the immune response and preparation of antibodies against a large panel of conjugated neurotransmitters and biogenic amines: specific polyclonal antibody response and tolerance

Han Huisman, Paul Wynveen[1] and Peter W. Setter[1]

Department for Research and Development, Pharmasan Labs Inc., Osceola, Wisconsin 54020, USA

Abstract

We described the production and characterization of antibodies against three important groups of neuro-active haptens, e.g., neurotransmitters and biogenic amines. First, from the tryptophane metabolic pathway: tryptamine, serotonin, 5-hydroxy-indole acetic acid, and melatonin. Secondly, the tyrosine metabolic pathway: tyramine, dopamine, dihydroxyphenyl acetic acid, and norepinephrine. Thirdly, antibodies against excitatory and inhibitory neurotransmitters: glycine, glutamate, glutamine, and GABA. Immunogenic conjugates were prepared after linking haptens to carrier proteins. Most antibodies displayed high specificity against corresponding neuro-active haptens conjugated *in vitro* and *in situ* in biological specimens, but not to closely related conjugated metabolites, precursors, pharmaceuticals, agonists, antagonists, or free neuro-active haptens. Conjugated norepinephrine was highly tolerant in different animal species and produced incidentally a short specific antibody response.

Keywords: biogenic amines, competitive ELISA, detection *in situ*, neurotransmitters, polyclonal antibodies, tolerance.

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It is known that neuro-active haptens such as neurotransmitters and biogenic amines are biologically active small molecules which have a systemic influence on the peripheral nervous system and are predominate actuators of the CNS (Cooper *et al.* 2003; Berry 2004; Eisenhofer *et al.* 2004). Analyses of these biologically active small molecules are indispensable for the elucidation of their (patho-) physiological roles. Neurobiology needs specific tools to attack the problem of neurotransmitter localization in tissue sections and the concentration during secretion and circulating in biological fluids. It is a fact that neurotransmitters and biogenic amines are either amino acids, biosynthesized derivatives of amino acids, or metabolites and cannot be detected as such by conventional immunological methods. It would be highly desirable to establish the concentration or the localization in biological matrices within a defined range to discriminate between normal good health and physical or mental disorder or disease.

Since the pioneering work of Landsteiner (1945), who raised antibodies to haptens, it has been possible to obtain specific antibodies to haptens such as amino acids, neurotransmitters, and biogenic amines (Habeeb and Hiramoto 1968; Avrameas 1969; Herzenberg *et al.* 1982; Geffard *et al.* 1985; Meyer *et al.* 1991). Antibodies are usually obtained after immunization of appropriate animals with conjugates consisting of the corresponding aminated-hapten linked with mono- or bivalent aldehydes to a suitable carrier protein (CP). The coupling mechanism for neurotransmitters to CPs used during the synthesis of the immunogen must preserve the distinguishing part of the molecule. Moreover, the immune response must direct selectively against the coupled hapten. Condensation of bi-functional aldehyde with neurotransmitters and CPs involves several parallel steps. In neutral aqueous solutions, the aldehyde group reacts with the non-ionized amino- or imine group to form a Schiff's base. Parallel aldol condensation of the second aldehyde group activated by the first one, stabilizes the Schiff's base (Kuznetsova *et al.* 2003). Antibodies can also be raised against de-aminated neuro-active haptens such as dihydroxyphenyl acetic acid (DOPAC), 5-hydroxyindole-acetic

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Address correspondence and reprint requests to Han Huisman, PhD, Pharmasan Labs Inc., 375, 280th Street Osceola, WI 54020, USA. E-mail: han.huisman@pharmasan.com

[1]These authors contributed equally to this study.

Abbreviations used: 5-HIAA, 5-hydroxyindole-acetic acid; BSA, bovine sera albumin.CP, carrier protein; DOPAC, dihydroxyphenyl acetic acid; KLH, keyhole limpet hemocyanin; MES, 2-(N-morpholino)ethanesulfonic acid; OVA, ovalbumin; PBS, phosphate-buffered saline; TG, thyroglobuline.

acid (5-HIAA), and melatonin after conjugation using the Mannich reaction (Hermanson 2008). The haptens are coupled to CPs via condensation of a (mono-)aldehyde with an active hydrogen atom present on tyrosine and tryptophane backbone. In the history of drug development to treat neurological disorders, precursors, agonists, and antagonists are often structural related molecules to a typical neurotransmitter such as Levodopa, Carbidopa, Phenibut (Lapin 2001; Turner *et al.* 2006; Khor and Hsu 2007; Kuoppamäki *et al.* 2009). To this end, we include these related structures to study the specificity of the antibodies in more detail. The antibodies described in this paper were analyzed for specificity with conjugated haptens as well as for detection of neuro-active haptens in biological specimens after conjugation *in situ*. We found that none of the antibodies were reactive with free unconjugated neuro-active haptens (cf. Hodgson *et al.* 1985; Campistron *et al.* 1986; Karasawa *et al.* 1992; Dabadie and Geffard 1993; Fujiwara *et al.* (1999) Vandenabeele-Trambouze *et al.* 2002; Minami *et al.* 2009).

The low but substantial cross-reactivity of antibodies against Catecholamines, suggests that at least three independent antigenic determinants are present on the tyrosine/catechol moiety. Antibodies against aliphatic amino acids with variable side chains: glycine, glutamine, and GABA, displayed high specificity suggesting that each hapten as a whole represents the optimal requirement for antibody binding. Different antibodies developed against Glutamate were all of moderate specificity and low titer, this result will be discussed in the context of extreme negative charge of the Glutamate conjugate. We demonstrate here that antibodies to metabolites of the tyrosine pathway, e.g., tyramine, dopamine, and DOPAC can be obtained with high titer and specificity. Dopamine, is the immediate precursor of norepinephrine after the hydroxylation of the side chain via dopamine-beta-hydroxylase (Eisenhofer *et al.* 2004). Multiple immunizations ($n = 12$) in rabbits, chickens and mice with conjugated norepinephrine resulted consequently in tolerance for this immunogen. In one occasion, a short but specific immune response against norepinephrine was observed which declined rapidly there after.

Here, we describe, in detail, the preparation of immunogenic conjugates, the specificity of the antibodies for conjugated neuro-active haptens, the ability to detect neuro-active haptens in clinical samples, and the putative antigenic determinants of the antibodies.

Materials and methods

Reagents

The following chemicals were purchased from Sigma Aldrich (Saint Louis, MO, USA): bovine serum albumin, ovalbumin (OVA), thyroglobuline (TG), gelatin from porcine skin (bloom 300) glutaric aldehyde, formaldehyde, GABA, L-glutamate, L-glutamine, L-aspartic acid, L-asparagine, L-glycine, L-alanine, L-leucine, L-valine, L-norvaline, L-ornithine, DL-5-hydroxylysine, 2-aminobutyric acid, L-glutamate-γ-methyl ester, L-glutamate-γ-ethyl ester, L-glutamate-γ-hydrazide, poly-L-glutamate, poly-L-glycine, 4-amino-3-(4-chlorophenyl)-butanoic acid (baclofen), *O*-phospho-L-serine, β-alanine, L-tryptophan, L-tryptamine, 5-hydroxy-DL-tryptophan, 5-hydroxytryptamine, 5-hydroxyindoleacetic acid (5-HIAA), 5-hydroxytryptophol, *N*-acetyl-5-hydroxytryptamine, melatonin, 6-hydroxymelatonin, DL-norepinephrine, DL-dopamine, DL-epinephrine, L-phenylalanine, L-tyrosine, L-dihydroxyphenylalanine, β-phenylethylamine, DL-tyramine, DL-octopamine, 3-hydroxy-4-methoxytyramine, 3-methoxytyramine, DL-nor-metanephrine, DL-metanephrine, *S*-(−)-carbidopa, 6-hydroxydopamine, dihydroxyphenylacetic acid (DOPAC), dihydroxyphenylglycol, goat-anti-rabbit IgG-alkaline phosphatase, and *p*-nitrophenyl phosphate disodium hexahydrate. L-Theanine was purchased from Taiyo International (Minneapolis, MN, USA). Keyhole limpet hemocyanin (KLH) was purchased from Pierce (Rockford, IL, USA). StabilGuard and StabilZyme Select were purchased from SurModics (Eden Prairie, MN, USA). Phenibut was purchased from KDN-Vita International, Inc. Hillsborough, NJ, USA.

All other chemicals were of analytical grade.

Preparation of hapten: protein conjugates

Immunogens-1

Amine containing neurotransmitters and biogenic amines (referred to as haptens for the remainder of this paper) were coupled to CPs: bovine sera albumin (BSA), TG from porcine, KLH, or OVA using the conventional one-step linking through glutaric aldehyde (Avrameas 1971; Geffard *et al.* 1985; Meyer *et al.* 1991). The cross-linking with CP's: BSA, TG, or OVA was performed in 100 mM Na-phosphate buffer pH 7.5 or in 50 mM sodium carbonate/bicarbonate buffer pH 9.2–9.6 both supplemented with 500 mM NaCl. Cross-linking with KLH was performed likewise but in the presence of 1.0 M NaCl throughout the whole procedure. The CP : hapten ratio was 10 : 1 (w/w), which is equal to, on average to 1 : 1 mM lysine to hapten ratio in case of BSA as CP and relatively close for TG, OVA, or KLH (Peters 1995; Hermanson 2008). Glutaric aldehyde concentration was between 5 and 10 mM in final. Incubation times varied between 30 min and 6 h at 25°C. Schiff's bases were reduced with a final concentration of 30 mM $NaCNBH_3$. Unbound haptens, free glutaric aldehyde, small polymers of glutaric aldehyde, and haptens were removed from the solution through 6000–8000 MWCO (Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA) dialysis against a 10 mM phosphate buffer (pH 7.5) with 500 mM NaCl, or with 1.0 M NaCl for KLH conjugates. Fine tuning to optimal incorporation of haptens were achieved using either pH 7.5 or 9.2, or small variations in glutaric aldehyde concentrations (see before) and CP : hapten (w/w) ratio from 10 : 1 to 10 : 0.1 (see before). Incorporation of haptens into CP was followed by spectrophotometric analyses, Isoelectric Focusing and sodium dodecyl sulfate–polyacrylamide gel electrophoresis, if appropriate. optimal CP-hapten immunogen was also assessed in a competitive ELISA with hapten-specific antibodies using haptens coupled to different CP. All immunogens were stored at final concentrations of 500 μg CP/mL with 0.02% NaN_3 at −20°C; except for KLH immunogens which were stored at +4°C.

Immunogens-2

Non-amine containing haptens: 5-HIAA, DOPAC, and melatonin were conjugated to CP through a Mannich condensation reaction (CP : hapten ratio, see before). Hapten-condensations were performed with a 3.5% final concentration of formaldehyde (with 10% methanol to prevent polymerization) in 0.25 M 2-(N-morpholino)ethanesulfonic acid (MES) buffer pH 5.5 containing 500 mM NaCl, 2 mM Vitamin C, and incubated for 24–48 h at 37°C protected from light. Unbound haptens, free formaldehyde, and other small fragments were removed by excessive dialysis through a 6000–8000 MWCO modified cellulose membrane (Spectro/Por, Spectrumlabs) against a 10 mM sodium hydrogen carbonate buffer pH 8.0 with 0.5 M NaCl. Fine tuning of the conjugated haptens were performed with slight modifications of CP–Hapten (w/w) ratio and incubation times, as monitored by spectrophotometric analysis, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, isoelectric focusing gel electrophoresis, if appropriate, and reactivity to hapten-specific antibody.

Hapten: protein conjugates for cross-reactivity studies

Each set of conjugated haptens for cross-reactivity studies were made under the same conditions as the homologous hapten conjugate. The glutaric aldehyde conjugates of aminated-haptens were made essentially according the procedure as described for immunogens, but only with BSA, TG, OVA, or Gelatin as CP. After terminating of the cross-linking reaction, unsaturated aldehyde groups were quenched with excess Tris (hydroxymethyl)aminomethane (Tris–HCl pH 7.5) at a final concentration of 10 mM and reduced with 30 mM $NaCNBH_3$. After dialysis in 10 mM Tris–HCl pH 7.5, 500 mM NaCl, the conjugated haptens were formulated to 1.0 mg CP/mL in 50% glycerol and 0.02% Na-azide and stored at −20°C. The formaldehyde Mannich Reaction conjugates were made essentially as described before, but only with BSA and OVA as CP, TG, and KLH are precipitated in the presence of formaldehyde at 3.5% final. The reaction was stopped by dialysis against 10 mM Tris–HCl pH 7.5, 500 mM NaCl. The Mannich condensation reaction conjugated haptens were formulated to 1.0 mg CP/mL in 50% glycerol and 0.02% Na-azide and stored at −20°C.

Immunization protocol

Immunization protocols were conducted at SynBioSci (Livermore, CA, USA) or Maine Biotechnology Service (Portland, ME, USA). New Zealand white rabbits were primed with 1 mL of hapten conjugate containing, on average, 50 μg of hapten coupled to 500 μg CP (see Results). The immunogen was mixed with 1 mL of Freund's complete adjuvant immediately prior to injection. The injections were administered subcutaneously in 8–10 sites on the flank and thorax of the rabbits. Booster injections were administered every 2 weeks and consisted of 1 mL of hapten conjugate containing on average 50 μg of hapten coupled to 500 μg CP and mixed with 1 mL of Freund's incomplete adjuvant immediately prior to injection. Serum was obtained 1 week after each booster for analysis of antibody specificity and titer. A typical protocol lasted 69 days, which resulted in six sequential bleeds, before the rabbits were exsanguinated. Prolonged immunizations were performed on successful immunizations with at least two to three other boosters every 2 weeks, before termination.

Antibody isolation

Based on the specificity and titer of the anti-hapten antibodies in whole serum, we determined whether further purification was necessary. If the whole serum exhibited cross-reactivity toward the homologous hapten and related molecules, an affinity-isolation was performed. If the whole serum exhibited reactivity only toward the homologous hapten and CP, an anti-CP antibody depletion was performed.

Hapten affinity columns were prepared using AminoLink Immobilization Kits (Pierce, Rockford, IL, USA) according to a modified protocol. AminoLink Coupling Resin (2 mL) was added to a 10-mL gravity column and equilibrated with a 0.1 M sodium borate buffer, pH 8.0 (Gentle Ag/Ab Binding Buffer; Pierce, Rockford, IL, USA). Ten mg of hapten was dissolved in 3 mL of sodium borate buffer and added to the column with 50 mM $NaCNBH_3$. Column was incubated in the dark with continuous rocking at 25°C for 6 h. Column was washed with excess 1.0 M Tris–HCl pH 7.5 containing 150 mM NaCl and quenched by incubating 3 mL of 1.0 M Tris pH 7.5, 50 mM $NaCNBH_3$ for 30 min at 25°C with constant rocking.

Antibodies were isolated from serum on a hapten affinity column according to the following protocol: Column was equilibrated with excess sodium borate buffer. De-lipidized serum was diluted 1 : 2 in sodium borate buffer and incubated on the column for 2 h at 25°C protected from light with constant rocking. After incubation, the column was washed with an excess of phosphate-buffered saline (PBS) until A_{280}nm was < 0.025. Antibodies were eluted with 0.1 M glycine-HCl, 250 mM NaCl pH 2.4 and immediately brought to pH 7 with Tris. Antibody solutions were dialyzed overnight against PBS. If appropriate, antibodies were isolated using a pH gradient from pH 6.0 to 1.5, to characterize antibody subpopulations with different specificities and affinity.

Carrier protein-glutaric aldehyde treated (CP-Cl) AminoLink columns were prepared as follows: glutaric aldehyde in a sub-optimal final concentration of 80 mM was added to 10 mg of CP in 3 mL 0.1 M PBS (BupH™ Phosphate Buffered Saline; Pierce) and allowed to incubate in the dark at 25°C for 60 min. One mL of 1.9 M ethanolamine pH 9.0 was added and the solution was incubated in dark at 25°C for 45 min. $NaCNBH_3$ was added to a final concentration of 50 mM. After an incubation of 15 min, the conjugate solution was then dialyzed extensively against 10 mM borate buffer pH 8.0 overnight at 4°C. The cross-linker-treated BSA (CP-Cl) was bound to AminoLink Coupling Resin according to the protocol before.

Carrier protein columns for antibodies raised against haptens coupled by the Mannich Condensation reaction were prepared using a PharmaLink Immobilization Kit (Pierce) according to a modified protocol. A PharmaLink column containing diaminodipropylamine coupled to 2 mL of agarose beads was equilibrated with 0.1 M MES, 0.15 M NaCl, pH 4.7. We then dissolved 10 mg of BSA in 4 mL of 0.1 M MES, 0.15 M NaCl, pH 4.7 and added formaldehyde containing 10% ethanol. Column was incubated overnight at 37°C with constant rocking. After incubation, the column was washed with an excess of 0.1 M Tris, pH 7.5.

Anti-CP antibodies were removed according to the following protocol: Columns were equilibrated with sodium borate buffer. Serum was diluted 1 : 2 in sodium borate buffer and incubated on the column for 1 h at 25°C with constant rocking. The flow through

of depleted serum was collected and anti-CP antibodies were eluted with 0.1 M glycine, pH 1.5 which was immediately neutralized with Tris. This process was repeated 2–3 times depending on the efficiency of the depletion of anti-CP-Cl antibodies. The completion of the depletion of anti-CP antibodies was assessed by a Direct ELISA. A Polystyrene 96 well flat-bottom immuno plates (Nalge Nunc International, Rochester, NY, USA) was coated with a the homologous and heterogeneous haptens coupled to different CPs. The ELISA for detection or absence of anti-CP was assessed essentially as described hereafter.

Competitive ELISA for cross-reactivity studies and direct ELISA for serum screening

A competitive ELISA was created for each anti-hapten antibody. Polystyrene 96 well flat-bottom immuno plates (Nalgene Nunc International) were coated overnight at 25°C and protected from light with the corresponding hapten conjugate in coating buffer (50 mM sodium carbonate-bicarbonate, pH 9.2–9.6) at a concentration optimized for each assay, usually between 1 and 5 μg CP/mL. Plates were washed six times with 250 μL/well of wash buffer (5 mM phosphate buffer pH 7.5, 50 mM NaCl, 0.002% Tween-20) using a Tecan 96 PW plate washer (Tecan Trading AG, Männedorf, Switzerland). This washing protocol was used after each incubation step. The plates were blocked with StabilGuard (SurModics) for at least 20 min. Standards were made by a serial dilution of homologous hapten conjugates to a heterologous CP in 10 mM sodium phosphate buffer pH 7.2 containing 150 mM NaCl (PBS), 10% StabilZyme Select (SurModics), 0.1% BSA, and 0.1% Tween-20. Standards and competitors (75 μL) were incubated along with (75 μL) optimized concentration of hapten-specific antibody in PBS, 0.1% BSA, and 0.1% Tween-20) overnight at 32°C with constant shaking. Plates were then incubated with 150 μL/well of secondary antibody, goat-anti-rabbit IgG-alkaline phosphatase, in PBS, 0.1% BSA, 0.5% normal goat serum, 0.1% Tween-20, for 1 h with constant shaking at 25°C at optimized concentrations. Finally, added 150 μL/well of alkaline phosphatase substrate buffer containing 2.5 mM *p*-nitrophenyl phosphate disodium hexahydrate in 1 M diethanolamine, pH 9.5. Absorbance at 405 nm was obtained using a Sunrise plate reader (Tecan).

Longitudinal serum bleeds were tested in a direct ELISA with serial dilutions on coated plates with the original immunogen and the homologous hapten conjugated to a heterologous CP. The coating of antigens, incubation time, washing of the plates, secondary antibody, and substrate development were as described before.

Determination of antibody specificity in a competitive ELISA

Antibody specificity was determined using the competitive ELISA and the results were the average of four independent observations. A standard titration of the conjugated hapten was prepared by serially diluting the 1 mg/mL CP in PBS, 0.1% BSA, and 0.1% Tween-20 and run in the ELISA under the above protocol. The related hapten conjugates were titrated under identical conditions and compared with the standard curve generated. The concentration of the hapten conjugate was plotted against the absorbance values obtained from the ELISA in a semi-log plot. After fitting the curve of the homologous hapten response, using a nonlinear regression, an equation was calculated from the linear portion of the curve. Observed concentrations for the related hapten conjugates were calculated from this equation using absorbance values which fell in the linear region of the standard curve. Cross-reactivity was expressed as the average ratio of observed concentration to actual concentration in relation to the homologous hapten. (EX. a 1 : 200 ratio conveys that a 200-fold increase in concentration of the related hapten conjugate is necessary to achieve the same response as the homologous conjugated hapten).

Detection of neurotransmitters in urine and plasma samples after conjugation with glutaric aldehyde *in situ*

Fresh collected urine samples, normalized with water to 40 mg/dL creatinine per sample, were spiked with a constant amount (100 ng/sample) of dopamine and glycine. The samples were adjusted to pH 8.0 with 1.0 M $NaHC0_3$. Glutaric aldehyde was added to a final concentration of 66 mM to cross-link endogenous amino-containing molecules, and spiked dopamine and glycine in urine samples. (cf. fixation of coupes in histochemistry). Excess glutaric aldehyde was quenched with excess of 1 M Tris–HCl pH 7.5 and the samples were analyzed in the competitive ELISA as described before. Control experiments with no cross-linking of the samples were also performed.

Plasma samples were spiked with constant amounts (100 ng/sample) of serotonin and GABA and de-proteinated with 5% cold trichloric acid with removal by centrifugation (10 000 *g*). The protein pellet was washed once with 0.5% trichloric acid and centrifuged again. Supernatants were combined and neutralized with KOH. To the de-proteinated plasma samples, 25 μg BSA was added followed by the addition of 100 mM final of glutaric aldehyde to cross-link endogenous amino-containing molecules and spiked serotonin and GABA. Excess Glutaric aldehyde was quenched with excess of 1 M Tris–HCl pH 7.5 and the samples were analyzed in the competitive ELISA as described before.

Results

Polyclonal antibody response in rabbits with 12 conjugated neuro-active haptens

We have generated 12 different neuro-active hapten polyclonal antibodies: (see Fig. 1) glutamate, glutamine, GABA, glycine, tyramine, dopamine, DOPAC, norepinephrine, tryptamine, serotonin, 5-HIAA, and melatonin. All 12 antibodies described here were successfully obtained with immunogens prepared according the one-step conjugation procedure with low hapten–carrier ratio. A typical immunization protocol lasts 69 days. After priming and second booster, the antibody titer and specificity for most of the 12 antibodies is at its maximum and remain until exsanguinations (c.f. Fig. 5a).

Immune response to serotonin was delayed and the maximal titer of high specific antibodies was obtained 3–4 months after starting the immunization. Antibodies to glutamate and norepinephrine were the only exceptions having a moderate specificity and a low titer.

None of the sera described in this paper were used unpurified, because of significant cross-reactivity, i.e., to CP and CP-Cl. Figure 2 shows the results of the characterization

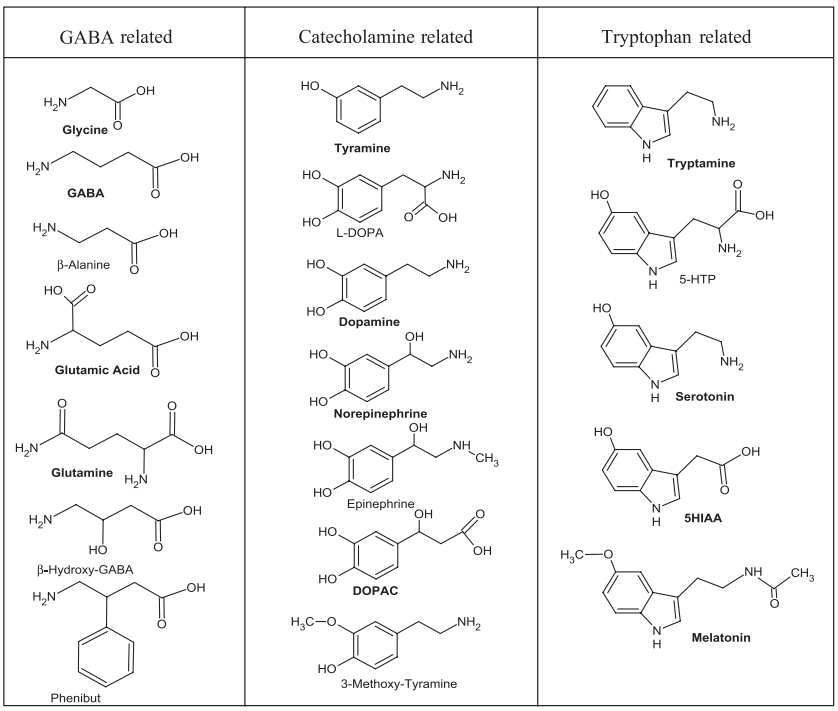

Fig. 1 Structures of the 12 neuroactive haptens in which specific antibodies were developed (bold and underlined) along with several other related molecules.

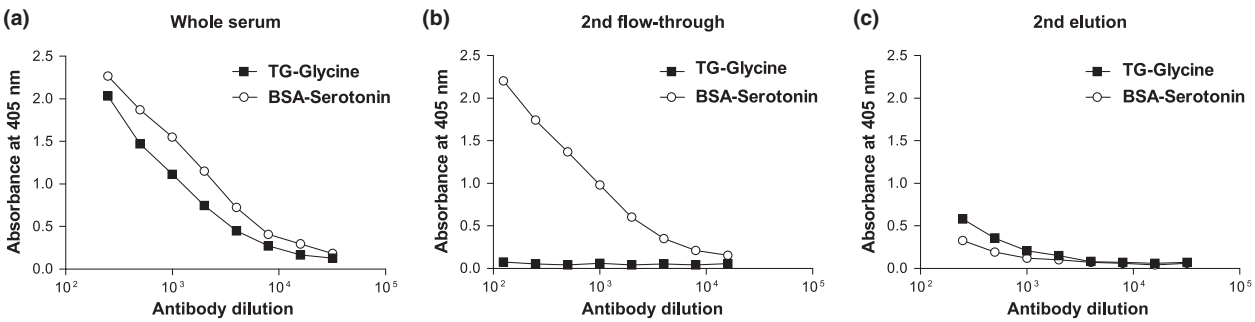

Fig. 2 Comparison of antibody specificity toward serotonin when depleted of TG-Cl-specific antibodies through affinity isolation. (a) Whole serum from rabbit immunized against a TG-serotonin immunogen. (b) Second flow-through. Serum passed twice over AminoLink-TG-column treated with glutaric aldehyde and quenched with ethanolamine. (c) Second elution. Bound antibodies after passing serum over the column twice (cf. b) and eluting with a glycine-HCl pH 1.5 elution buffer. All fractions were measured against TG-glycine and BSA-serotonin coated on ELISA plates.

and purification of antibodies against serotonin. It is a representative example to obtain purified antibodies by comparing sera before and after depletion of cross-reacting antibodies with CP and glutaric aldehyde moieties (CP-Cl) (Fig. 2a–c).

Depletion of CP- and CP-Cl-specific antibodies is not always sufficient to abolish cross-reactivity to related haptens as became apparent for Dopamine antibodies. Figure 3 displayed the results obtained with affinity-purified antibodies against dopamine and the characterization of subsets of antibodies isolated via a pH gradient. Antibodies isolated at pH 6.0, 5.0, and 3.5, cross-react slightly with norepinephrine (Fig. 2a–c). However, antibodies isolated finally at pH 2.4 and 1.5 (the latter not shown), reacting exclusively with dopamine only (Fig. 3d). Other affinity-purified antibodies (cf. Table 1) did not contain subsets of antibodies with mixed specificity.

The purified antibodies specific for GABA (immunogen: BSA-GABA); glutamine (immunogen: KLH-Glutamine) and glycine (immunogen: KLH-Glycine) were tested in their response to haptens conjugated to CPs different from the original immunogen, e.g., BSA, TG, OVA, and KLH. The result was that the specificity to the homologous-conjugated hapten remained the same, irrespective the choice of the CP, see Figure 4. The same results were found for the other purified antibodies (data not shown).

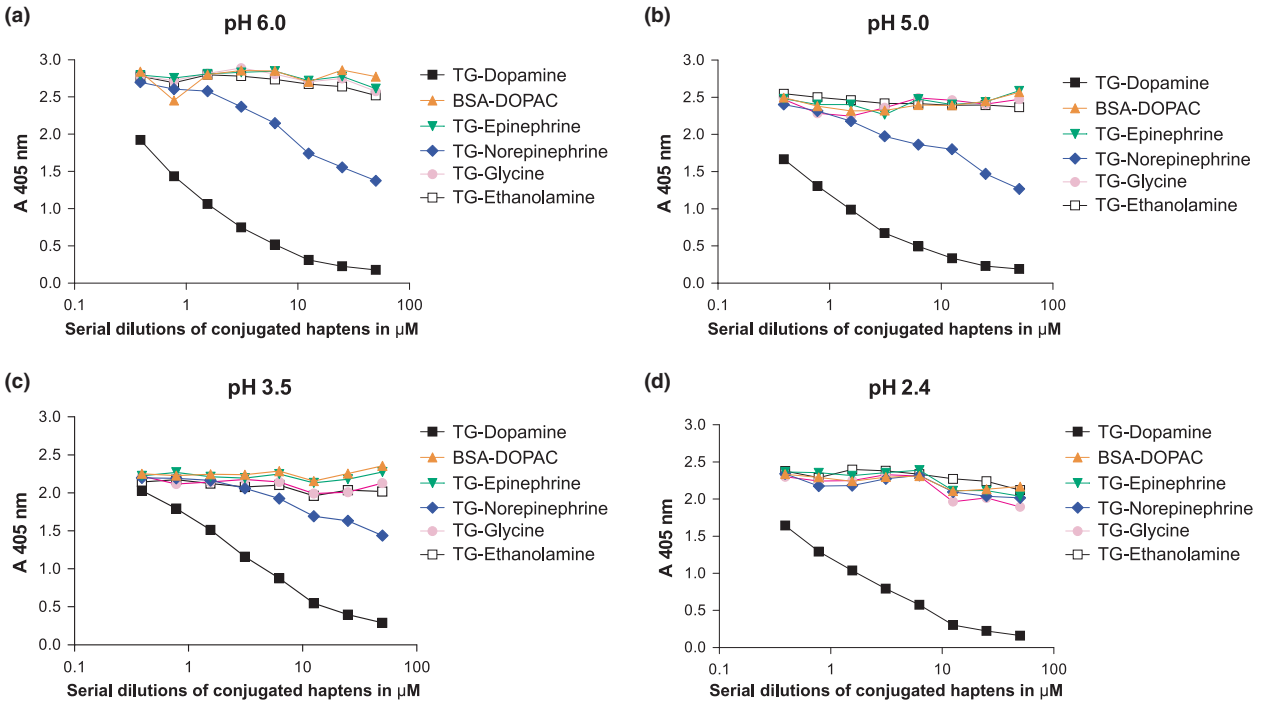

Fig. 3 Comparison of the specificity of anti-dopamine antibodies isolated by affinity chorography and pH gradient. Anti-dopamine antibodies were bound to a column of dopamine conjugated to AminoLink beads and eluted consecutively with a pH gradient of pH 6.0 (a), 5.0 (b), 3.5 (c), and 2.4 (d). Reactivity was measured in a competitive ELISA against conjugated dopamine, DOPAC, epinephrine, norepinephrine, and glycine. BSA-conjugated dopamine was coated on ELISA plates. None of the antibodies were reacting with non-conjugated haptens.

Table 1 Composition of each immunogen used for successful immunization of two rabbits or multiple of two. Depicted is also the optimal purification of each antibody for cross-reactivity studies

Immunogen (cross-linker)	Immunization	Optimal purified antibody
BSA-GABA (glutaric aldehyde)	1 × 2 rabbits	Affinity purified IgG-GABA
KLH-glycin (glutaric aldehyde)	1 × 2 rabbits	Affinity purified IgG-Glycin
BSA/KLH-glutamate (glutaric aldehyde)	3 × 2 rabbits	IgG-glutamate depleted for CP-Cl
KLH-glutamine (glutaric aldehyde)	1 × 2 rabbits	Affinity purified IgG-glutamine
BSA-tryptamine (glutaric aldehyde)	1 × 2 rabbits	IgG-tryptamine depleted for CP-Cl
TG-serotonin (glutaric aldehyde)	2 × 2 rabbits	IgG-serotonin depleted for CP-Cl
BSA-5-HIAA (Mannich reaction with formaldehyde)	1 × 2 rabbits	IgG-5-HIAA depleted for CP-Cl
BSA-melatonin (Mannich reaction with formaldehyde)	1 × 2 rabbits	IgG-melatonin depleted for CP
BSA-tyramine (glutaric aldehyde)	1 × 2 rabbits	IgG-tyramine depleted for CP-Cl
BSA-dopamine (glutaric aldehyde)	2 × 2 rabbits	Affinity and IgG-dopamine depleted for CP-Cl
BSA-DOPAC (Mannich reaction with formaldehyde)	2 × 2 rabbits	IgG-DOPAC depleted for CP
BSA/TG/KLH/cBSA norepinephrine (glutaric aldehyde and EDC)	Multiple immunizations in either rabbits, mice, or chickens	Affinity and IgG-norepinephrine depleted for CP-Cl

In Table 1, we have summarized the type of immunogen, the number of rabbits used for a successful immunization and the purification requirement, e.g. affinity purification or depletion for CP/glutaric aldehyde reactivity, for optimal test results.

Cross-reactivity study of three groups of neuro-active hapten antibodies

The results of cross-reactivity studies with conjugated homologous and heterologous haptens presented in this paper are the average of at least four independent experi-

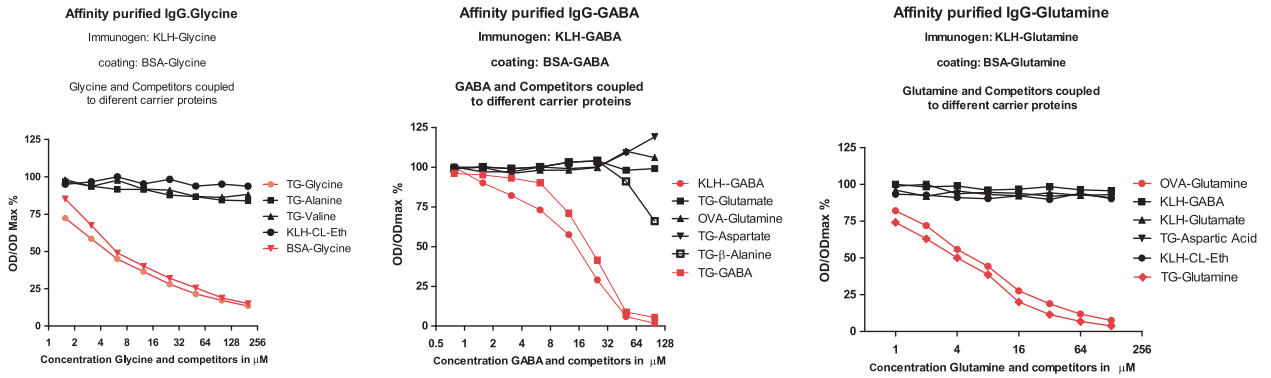

Fig. 4 Affinity purified IgG against GABA (immunogen: BSA-GABA); glycine (immunogen: KLH-Glycine), and glutamine (immunogen: KLH-Glutamine) were tested in a competitive ELISA for their reactivity to homologous and heterologous immunogens conjugated to a variety of carrier proteins: KLH, BSA, OVA, and TG. None of the antibodies react with non-conjugated haptens.

ments; variability data are included in the figures and tables, if appropriate. None of the antibodies reacted with free neuro-active haptens.

Glutamate, glutamine, GABA, and glycine

Table 2 summarizes the reactivity of purified antibodies (cf. Table 1) raised against conjugated glutamine, GABA, and glycine with large panel of conjugated charged amino acids, related chemical structures, pharmaceuticals antagonists, and agonists. All three antibodies displayed unequaled high specificity. Antibodies to glutamate displayed moderate specificity (Table 2).

Tryptamine, serotonin, 5-HIAA, melatonin

In Table 3, we have summarized the specificity of these antibodies derived from four metabolites of the tryptophane pathway. All antibodies displayed high specificity with no detectable reactivity with other derivatives. Melatonin and 5-

Table 2 Specificity of anti-glutamate, anti-glutamine, anti-GABA, and anti-glycine antibodies

Competitor	Anti-glutamate	Anti-glutamine	Anti-GABA	Anti-glycine
BSA-Glutamate	1	> 1 : 10,000	> 1 : 10,000	> 1 : 10,000
BSA-Glutamine	1 : 31	1	> 1 : 10,000	> 1 : 10,000
BSA-GABA	1 : 40	> 1 : 10,000	1	> 1 : 10,000
BSA-Glycine	> 1 : 10,000	> 1 : 10,000	> 1 : 10,000	1
BSA-Aspartic Acid	1 : 20	> 1 : 10,000	N/A	> 1 : 10,000
BSA-Asparagine	1 : 20	> 1 : 10,000	N/A	> 1 : 10,000
BSA-Norvaline	1 : 40	> 1 : 10,000	N/A	> 1 : 10,000
BSA-Ornithine	1 : 45	> 1 : 10,000	N/A	> 1 : 10,000
BSA-5-Hydroxylysine	1 : 52	> 1 : 10,000	N/A	> 1 : 10,000
BSA-L-Theanine	1 : 43	> 1 : 10,000	> 1 : 10,000	> 1 : 10,000
BSA-O-Phospho-L-Serine	1 : 23	> 1 : 10,000	N/A	> 1 : 10,000
BSA-2-aminobutyric acid	1 : 40	> 1 : 10,000	> 1 : 10,000	> 1 : 10,000
BSA-Glutamate-γ-methyl ester	1 : 13	> 1 : 10,000	> 1 : 10,000	> 1 : 10,000
BSA-Glutamate-γ-ethyl ester	1 : 7	> 1 : 10,000	> 1 : 10,000	> 1 : 10,000
BSA-Glutamate-γ-hydrazide	1 : 63	> 1 : 10,000	N/A	> 1 : 10,000
BSA-poly-glutamate (50-100 kDa)	1 : 42	> 1 : 10,000	N/A	N/A
BSA-poly-glutamate (700-4000 kDa)	1 : 42	> 1 : 10,000	N/A	N/A
BSA-Poly-Glycine	1 : 49	> 1 : 10,000	N/A	N/A
BSA-Phenibut	N/A	N/A	> 1 : 10,000	> 1 : 10,000
BSA-Baclofan	N/A	N/A	> 1 : 10,000	> 1 : 10,000
BSA-β-Alanine	N/A	N/A	1 : 77	> 1 : 10,000

The specificity toward a conjugate was calculated by comparing the observed concentration with the actual concentration. (cf. Fig. 1). None of the antibodies react with free haptens. N/A, not analyzed (n = 4; %CV = 2.2%–5.1%). Cross-reactivity of glutamate, glutamine, GABA, and glycine antibodies with related conjugated competitors.

Table 3 Specificity of anti-serotonin, anti-tryptamine, anti-5-HIAA, and anti-melatonin antibodies

Competitor	Anti-serotonin	Anti-tryptamine	Anti-5-HIAA	Anti-melatonin
TG-tryptophane	> 1 : 10 000	> 1 : 10 000	> 1 : 10 000	> 1 : 10 000
BSA-tryptamine	1 : 3 125	1	> 1 : 10 000	> 1 : 10 000
BSA-5-HTP	> 1 : 10 000	> 1 : 10 000	> 1 : 10 000	> 1 : 10 000
BSA-5-HT	1	1 : 5 000	> 1 : 10 000	> 1 : 10 000
BSA-5-HIAA	> 1 : 10 000	> 1 : 10 000	1	> 1 : 10 000
BSA-5-HTOL	> 1 : 10 000	> 1 : 10 000	> 1 : 10 000	> 1 : 10 000
BSA-N-Ac-5-HT	> 1 : 10 000	> 1 : 10 000	> 1 : 10 000	> 1 : 10 000
BSA-melatonin	> 1 : 10 000	> 1 : 10 000	> 1 : 10 000	1

The specificity toward a conjugate was calculated by comparing the observed concentration with the actual concentration (cf. Fig. 1). None of the antibodies react with free haptens ($n = 4$; %CV = 2.6%–4.8%). Cross-reactivity of serotonin, tryptamine, 5-HIAA, and melatonin antibodies with related conjugated competitors.
5-HT, 5-hydroxytryptamine; 5-HTOL, 5-hydroxytryptophol; 5-HTP, 5-hydroxy-DL-tryptophan; *N*-Ac-5-HT, *N*-acetyl-5-hydroxytryptamine.

HIAA conjugates were made according the Mannich reaction (see Materials and methods).

Tyramine, dopamine, DOPAC, norepinephrine

Table 4 summarizes the characterization of these antibodies derived from the tyrosine pathway. To our knowledge we describe here for the first time polyclonal antibodies against DOPAC. The DOPAC antibody displayed a very high specificity. Non-fractionated affinity purified antibodies against dopamine and norepinephrine displayed a small but detectable cross-reactivity to each other (1 : 40 and 1 : 47, respectively, cf. Fig. 3).

Immune tolerance or suppression to conjugated norepinephrine?

The described specific antibody in a rabbit against norepinephrine (immunogen-BSA-Norepinephrine) was a one-time success after numerous immunizations in different animal species with different conjugation procedures, e.g., glutaric aldehyde and carbodiimide. Figure 5(b) displays the immune response against conjugated morepinephrine in four sequential bleeds. Initially, the highest antibody response is against norepinephrine if compared with dopamine and CP-Cl. In the final bleed, the titer has dropped as well as the specificity. As a representative example for all other

Table 4 Specificity of anti-norepinephrine, anti-dopamine, anti-DOPAC, and anti-tyramine antibodies

Competitor	Anti-norepinephrine	Anti-dopamine	Anti-DOPAC	Anti-tyramine
BSA-norepinephrine	1	1 : 40	> 1 : 10 000	> 1 : 10 000
BSA-dopamine	1 : 47	1	> 1 : 10 000	> 1 : 10 000
BSA-epinephrine	1 : 263	> 1 : 10 000	> 1 : 10 000	> 1 : 10 000
BSA-phenylalanine	> 1 : 10 000	> 1 : 10 000	> 1 : 10 000	> 1 : 10 000
BSA-tyrosine	> 1 : 10 000	> 1 : 10 000	> 1 : 10 000	> 1 : 10 000
BSA-L-DOPA	> 1 : 10 000	> 1 : 10 000	> 1 : 10 000	> 1 : 10 000
BSA-β-PEA	1 : 270	1 : 3333	> 1 : 10 000	> 1 : 10 000
BSA-tyramine	1 : 357	> 1 : 10 000	> 1 : 10 000	1
BSA-octopamine	1 : 40	> 1 : 10 000	> 1 : 10 000	N/A
BSA-3-hydroxy-4-methoxy-tyramine	1 : 833	1 : 200	> 1 : 10 000	N/A
BSA-3-methoxy-tyramine	> 1 : 10 000	1 : 38	> 1 : 10 000	> 1 : 10 000
BSA-normetanephrine	1 : 244	> 1 : 10 000	> 1 : 10 000	> 1 : 10 000
BSA-metanephrine	> 1 : 10 000	> 1 : 10 000	> 1 : 10 000	> 1 : 10 000
BSA-carbidopa	> 1 : 10 000	1 : 5000	> 1 : 10 000	N/A
BSA-6-hydroxydopamine	> 1 : 10 000	> 1 : 10 000	> 1 : 10 000	N/A
BSA-DOPAC	> 1 : 10 000	> 1 : 10 000	1	> 1 : 10 000
BSA-DHPG	> 1 : 10 000	> 1 : 10 000	> 1 : 10 000	> 1 : 10 000

The specificity toward a conjugate was calculated by comparing the observed concentration with the actual concentration (cf. Fig. 1). None of the antibodies react with free haptens. N/A, not analyzed ($n = 4$, %CV = 3.8%–7.9%). Cross-reactivity of norepinephrine, dopamine, DOPAC, and tyramine antibodies with related conjugated competitors.
DHPG, dihydroxyphenylglycol; β-phenylethylamine (β-PEA); DOPAC, dihydroxyphenyl acetic acid.

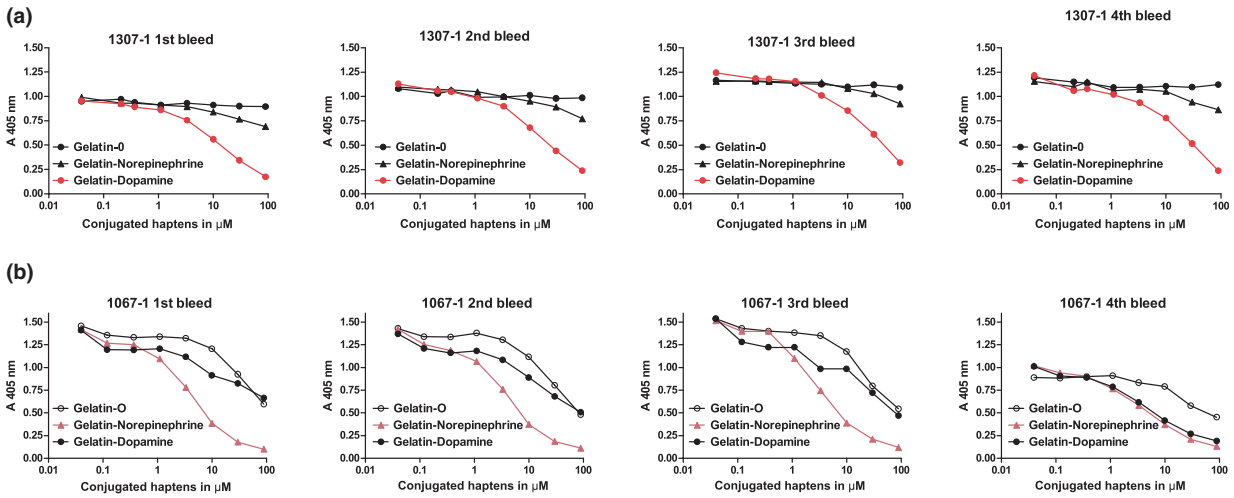

Fig. 5 Specificity and titer of dopamine and norepinephrine antibodies over the course of an immunization protocol. (Sequential bleeds were obtained every 2 weeks after the first booster injection). (a) Anti-dopamine antibodies depleted from BSA-Cl antibodies of rabbit serum immunized against BSA-dopamine. (b) Anti-norepinephrine antibodies depleted from BSA-Cl antibodies of rabbit serum immunized against BSA-norepinephrine. In the competitive ELISA, plates were coated either with BSA-dopamine (a) or BSA-norepinephrine (b). Competitors see: legends sequential bleeds from dopamine and norepinephrine.

antibodies we show in Figure 5(a), the immune response to another catecholamine, e.g., dopamine exhibiting a specific antibody response preferentially to dopamine with a constant relative high titer over time.

Detection of neurotransmitters in biological specimens after conjugation *in situ* using a competitive ELISA

In Figure 6, we show the presence of endogenous neurotransmitters glycine and dopamine conjugated *in situ* in 12 urine samples as well as GABA and serotonin conjugated *in situ* in eight de-proteinated plasma samples (Davis 1989). Each neurotransmitter has its own specific distribution pattern in the biological specimen and can be detected only with the homologous antibody (cf. Fig. 6, a1–2 and b1–2). As apparent from Figure 6(a1–2), the neurotransmitters glycine and dopamine, spiked and endogenous, were only detectable with cross-linking *in situ*. In addition, only the homologous antibodies were able to detect in a relative quantitative way the spiked neurotransmitters in the urine and plasma samples. The values are expressed as arbitrary units. A manuscript is in preparation of (semi-) quantitative measurements of neurotransmitters in relation to clinical disorders.

Discussion

One-step conjugation

We have used the one-step procedure with low glutaric aldehyde concentration to achieve predominantly Schiff base formation and less glutaric aldehyde oligomerization (Kuznetsova *et al.* 2003. The amount of on average 1 mM of hapten coupled to 1 mM of lysine in the CP-BSA we have been using, is much lower (factor > 50) to what has been described in the literature (Geffard *et al.* 1985; Meyer *et al.* 1991). We speculate that a lower density of bound haptens might be important to maintain the chemical integrity and as such the epitope of the hapten in the background of the CP (Pedersen *et al.* 2006; Singh *et al.* 2004). Any statement made about the exposure of the hapten from the CP is speculative.

For non-amine-containing haptens, the Mannich Reaction appeared to be a reliable procedure. Via aldehyde, the condensation reaction occurred between the available amine-groups on the CP and the active hydrogen on either the tyrosine or tryptophane backbone (Hermanson 2008).

Comparison serum, depleted serum and affinity purified antibodies and reactivity to different CPs

The specificity of hapten antibodies are very often obscured by interfering subpopulations of antibodies reacting with either, the CP, aldehyde moiety, oligomers of glutaric aldehyde, or combinations thereof. In addition, binding of antibodies could be virtually absent to haptens presented in a fluid phase assay but detectable in a solid phase assay, because of higher density and immobilization of the antigens on the solid phase. In Figure 2, we described the successful depletion of interfering TG-Cl-antibodies from serum of immunized rabbits with TG-serotonin. The immunizations with conjugated metabolites of the tryptophane pathway were all very successful with high specificity and titer. The absence of cross-reactivity to immobilized competitor is important as most of neuro-active antibodies are applied for localization of neuro-active haptens in mono- or dialdehyde fixed tissues (Seguela *et al.* 1984; Luther and Bloch 1989;

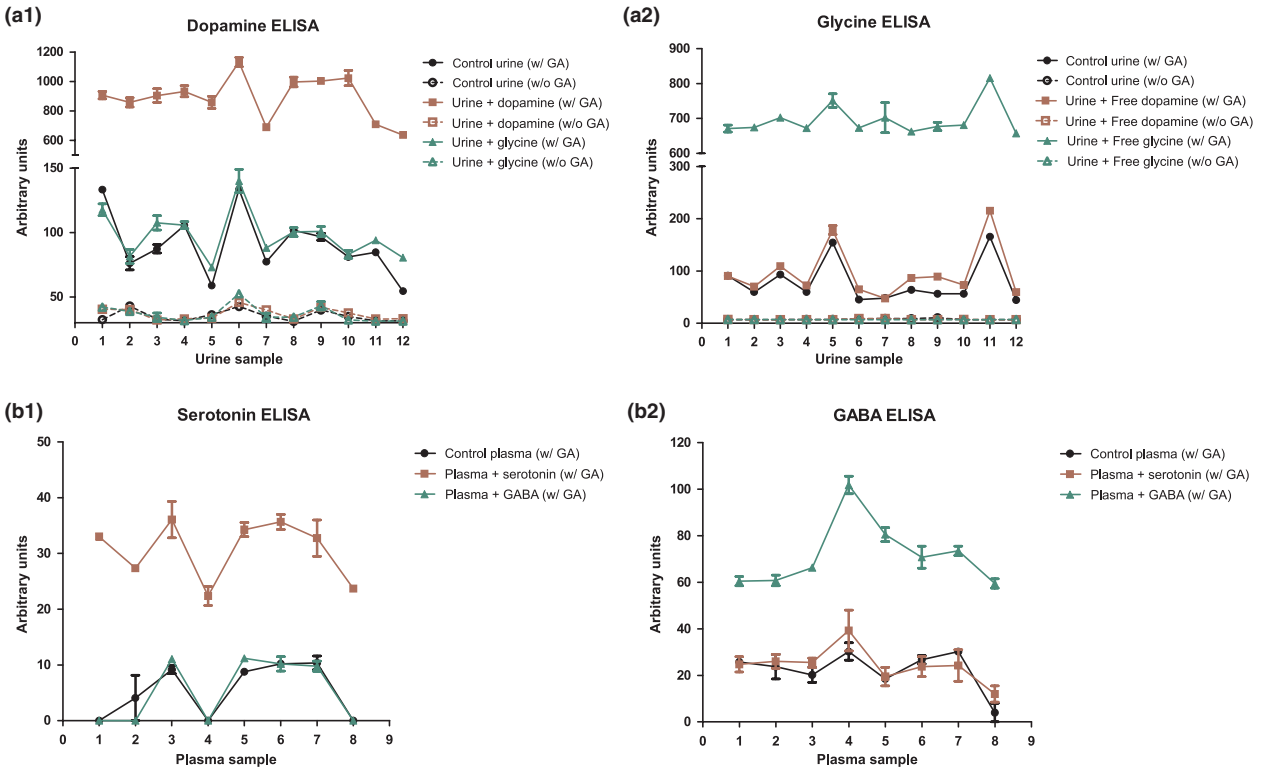

Fig. 6 Detection of neurotransmitters in biological specimens after *in situ* conjugation and using specific antibodies in the competitive ELISA format. (a) Dopamine and glycine-specific ELISA analysis of 12 urine samples with and without spiking of free dopamine or glycine, with and without glutaric aldehyde cross-linking. (b) Serotonin and GABA-specific ELISA analysis of eight plasma samples, with and without spiking of free serotonin or GABA, treated with glutaric aldehyde after de-proteination. (GA = glutaric aldehyde; w/GA = with GA; w/o GA = without GA). Standard error: urine, ≤ 4.7%; plasma, ≤ 14.5%.

Campistron *et al.* 1986), or in competitive ELISA for measuring conjugated haptens in biological fluids (see Fig. 6, manuscripts in preparation, and Kim *et al.* 2008). Affinity purified antibodies against dopamine appeared to be a mixture of antibodies, the majority specific for dopamine (cf. Fig. 3). A relative small sub-population of these antibodies was also reactive with norepinephrine as well (cross-reactivity: 1 : 40). This subset of antibodies was identified and isolated via an affinity column using a pH gradient of elution buffer. Because of the pH shift from neutral to pH 6.0, 5.0, and 3.5, the antibody binding site and the immobilized hapten become more charged and repel each other. We consider that antibodies eluted at pH between 6.0 and 3.5 are indicative for a subset of lower affinity and less specific antibody. Only at pH 2.4 and lower, the antibody with the highest affinity because of the best fit can be dissociated from the hapten-column.

As demonstrated in Figure 5, the specificity of the antibodies is independent to which CP the hapten has been conjugated. Obviously, we have observed small quantitative differences in reactivity to the hapten bound to the different CPs caused by the number and susceptibility of available lysine molecules in the different CPs (Hermanson 2008). For cross-reactivity studies with depleted and/or affinity purified antibodies (see below), we prepared all competitor BSA-conjugates under strict comparable conditions to achieve comparable incorporation rates.

Characterization of antibodies against glutamate, glutamine, GABA, and glycine

The high specificity of the GABA, glutamine, and glycine antibodies to a large panel of related molecules argues for an antigenic determinant equal to the entire structure of the conjugated hapten. None of the antibodies recognized free hapten molecules (not shown). Each slight modification of the basic structure, as represented by the large number of related molecules, including GABA mimetics: Phenibut and GABA-OH, resulted in loss of antibody binding. There is one exception for the GABA antibody displaying a very small cross-reactivity 1 : 77, (Table 2 and Fig. 5) with β-Alanine, structurally identical to the de-carboxylation product of aspartic acid, as GABA is from glutamic acid. β-Alanine and GABA are similar in structure except for an extra CH_2 insert for GABA (cf. Fig. 1).

The formation of saturated Schiff bonds with $NaCNBH_3$ (Sodium Cyanogen Borohydride) is an integral part of the

conjugation reaction for preparing immunogenic conjugates and competitors. All antibodies described in this paper were only slightly sensitive (< 5%) to unsaturated Schiff's bonds. Glutamine antibody was an exception; it does not bind to a glutamine conjugate without reduction of Schiff's bonds (data not shown). The glutamate antibodies displayed a moderate specificity. First, the presence of two carboxylic groups at the alpha and y-positions can substantially decelerate conversion of this acid into nucleophilic agent with non-ionized amino-groups. Consequently, the conjugation rate of glutamate into CPs might be insufficient, to serve as a strong immunogen. However, we are speculating that the heavy negative charge of the conjugated glutamate immunogen might be disadvantageous for proper presentation to antigen presenting cells. In studies performed by Maurer and Sela on antigenicity of synthetic poly amino acids, they also mentioned the poor immunogenicity of poly amino acids containing glutamic acid in combination with other poly amino acids. (cf. Maurer 1964; Fuchs and Sela 1963).

Characterization of antibodies against tryptamine, serotonin, 5-HIAA, and melatonin

According to the metabolic pathway of Tryptophane (Kema *et al.* 2000), there is a central role for the indole nucleus as carrier for immunogenic lateral chains. The high specificity of the four antibodies indicates that epitopes of the antibodies are determined by each specific side chain. For melatonin, the specificity depends on the presence of 5-methoxyindole group and the non-substituted nucleus at the position 6 (OH for 6-hydroxymelatonin) (Minami *et al.* 2009; Dabadie and Geffard 1993; Harthé *et al.* 1991). For tryptamine and 5-hydroxytryptamine (serotonin), the antibody specificity depends on the non-substituted nucleus at position 5 (OH) for the tryptamine antibody, the OH-substitution is a prerequisite for the epitope of the serotonin antibody.

5-Hydroxyindole-acetic acid differs significantly from serotonin after de-amination and aldehyde dehydrogenase of the amino group into a carboxylic acid, which explains the high specificity of this antibody (cf. Fig. 1).

Characterization of tyramine, dopamine, DOPAC, and norepinephrine

Dopamine, 3,4-dihydroxyphenylethylamine differs from norepinephrine 3,4-dihydroxybenzylalcohol in a hydroxyl group in the side chain (see Fig. 1). This determines the key difference between these two neurotransmitters and antibody specificity (Mons and Geffard 1987). Cross-reacting antibodies can be removed by either carefully dissociating antibodies from a hapten-column at a pH gradient (see Fig. 3) or depletion of antibodies over a norepinephrine column. This results in gradual increase of the specificity for dopamine with a simultaneous drop in the overall titer (data not shown). Both antibodies are non-reactive with epinephrine (3,4-dihydroxymethyl amino methyl benzyl alcohol). This is obvious for dopamine antibodies. Dopamine differs at three positions with epinephrine; the hydroxyl group and amine group changed into a methylated imine group (> 1 : 10 000). Norepinephrine differs at two positions with epinephrine and displayed 1 : 263 reactivity to conjugated epinephrine (cf. Fig. 1). Norepinephrine antibodies displayed low but detectable reactivity against the products of the catechol-*ortho*-methyltransferase conversion, 1 : 244 (Table 3). In summary, antibodies to the catecholamines contain detectable overlapping specificities which at least three different epitopes: the 3,4-dihydroxy group in conjunction with either the absence of the OH-group (dopamine) or presence of the OH in the side chain, 3,4-dihydroxybenzylalcohol (norepinephrine); and the methylated imine group for epinephrine.

Tolerance or induction of suppression for norepinephrine specific antibody response

The low success rate for raising norepinephrine antibodies could not be ascribed to inconsistent stoichiometric of the making of these immunogens since hapten conjugates were made with a variety of CPs and different molar ratios. It is known that catecholamines are prone to (auto-) oxidation (Smythies and Galzigna 1998). We have investigated if norepinephrine sera might have contained a myriad of antibodies against degradation products derived from the original immunogen. We have found that conjugated catecholamines are moderate stable compared with the free molecule. Induced oxidized norepinephrine and dopamine at variable pHs results in a mixture of catecholamine-quiniones and aminochromes (Heacock and Hutzinger 1968). After conjugation, these molecules did not react with dopamine or norepinephrine serum bleeds (not shown). The norepinephrine immunizations ($n = 12$) resulted in the following observations: rabbits, chicken, or mice were either entirely non-responders, highly reactive to CPs and negative to the catecholamine or with minimal amounts of antibodies cross-reacting with all catecholamines and metabolites.

For norepinephrine, a specific immune response was obtained once with a good titer and moderate specificity but dropped at the end of the immunization period (see Table 3 and Fig. 5b). We have not observed this for the other antibodies, this is demonstrated with the immune response to dopamine as a representative example (cf. Fig. 5a). We cannot rule out that immunization procedure for norepinephrine antibodies needs specific requirements to avoid premature degradation of conjugated norepinephrine for developing good antibodies (Mons and Geffard 1987). Alternatively, in the case of norepinephrine, the carrier–protein–hapten-specific regulatory system could selectively induced to suppress primary and secondary antibody responses to hapten without interfering with antibody responses to epitopes on the carrier molecule. (Herzenberg and Tokuhisa 1962; McDevitt and Sela 1967; Schwartz *et al.* 1979).

Detection of neurotransmitters in biological specimens

From each of the three groups of antibodies presented in the paper, we have selected the GABA and glycine antibodies (Table 2), serotonin antibody (Table 3), and dopamine antibody (Table 4), to detect the corresponding neurotransmitter in either urine or plasma. The amount of these neurotransmitters in plasma and urine are in high ng/mL range (Davis 1989). The detection of the four neurotransmitters, as expressed in arbitrary units, is dependent on the cross-linking of the neurotransmitters in the biological samples (Fig. 6, A-1-2) and the use of the homologous antibody in the corresponding ELISA (Fig. 6, A1-2 and B1-2). Each neurotransmitter displayed its own specific profile in both urine and plasma samples, which remains proportional between spiked and endogenous conditions. The semi-quantitative recovery of the spiked neurotransmitters in urine and plasma demonstrate again the specificity and ability of the antibody to detect the homologous neurotransmitter after conjugation *in situ.* A manuscript is in preparation on the semi-quantitative measurement of neurotransmitters in clinical samples.

In summary, the successful preparation of immunogenic conjugates was based on low hapten–carrier ratio and sub-optimal amounts of cross-linker. This resulted in very specific antibodies for the haptens belonging to the tryptophan pathway and group of excitatory and inhibitory neurotransmitters and biogenic amines. For these antibodies the hapten-specificity was almost synonymous with the epitope structure. Antibodies elicited to the catecholamines: dopamine, DOPAC and one-time against norepinephrine displayed good specificity. These antibodies recognize preferentially small variation within the catechol moiety, the lateral 3,4-dihydroxy side chain seemed to be unimportant. We used a subset of antibodies specific for GABA, glycine, dopamine, and serotonin to prove that we were able to detect endogenous and spiked homologous neurotransmitters in urine and serum only after conjugation *in situ.*

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