Specificity of Chicken (IgY) versus Rabbit (IgG) Antibodies Raised against Cholecystokinin Octapeptide (CCK-8)

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Summary

Antibodies produced in chickens (egg yolk antibody-IgY) and rabbits against CCK-8 TyrSE (a C-terminal extended CCK-version) were compared with respect to their specificity against several modified CCK-sequences by means of radioimmunoassay and spot blot assay. The content of neuronal CCK was determined by using both an „avian" and a „mammalian" RIA. The IC_{50} values obtained indicate differences between the binding capacity of rabbit and chicken antibody, respectively. Supported by the data from spot blot assay, it appears, that the avian antibody binding activity was directed primarily towards short CCK-sequences whereas the longer sequences are less well recognised in contrast to the mammalian antibody. Probably, these differences may be due to characteristics regarding the shape of the molecules (caused also by fixation processes necessary for blotting procedures) as well as to structural differences between avian and mammalian antibodies itself (both antibodies originate from quite different immune systems). By comparing the quantitative CCK data (avian versus mammalian RIA) a significant correlation could be observed. Immunohistochemical studies using avian antibodies revealed a neuronal CCK pattern different from those using rabbit antibodies. These results are discussed on the basis of the specificity studies.

Keywords: CCK, IgY, egg yolk antibody, RIA, alternative

Zusammenfassung: Spezifitätsvergleich zwischen Hühner (IgY)- und Kaninchen (IgG)-Antikörpern gegen das Cholezystokinin Oktapeptid CCK-8.

Antikörper gegen CCK-8 TyrSE (eine CCK-Variante mit C-terminaler Extension) wurden sowohl im Huhn (Extraktion der Antikörper aus dem Dotter (IgY-Antikörper) als auch im Kaninchen erzeugt. Die resultierenden Antikörper wurden hinsichtlich ihrer Bindung mit verschieden modifizierten CCK-Sequenzen verglichen. Die Vergleiche erfolgten mittels Radioimmunassay (RIA) und Spot-Blot-Assay. Anhand von CCK-Gehaltsbestimmungen sollte die Spezifität beider Antikörper zusätzlich verglichen werden. Die demonstrierten IC_{50} Werte weisen auf Unterschiede im Bindungsverhalten beider Antikörper hin. Während der Huhn-Antikörper anscheinend kurze CCK-Sequenzen besser „erkennt" und differenziert, ergeben sich kaum Unterschiede zwischen den IC_{50} Werten bei den längeren Sequenzen. Auch im Spot-Blot-Assay wurde CCK-4 wesentlich deutlicher detektiert als CCK-8, im Unterschied zu dem Kaninchen Antikörper. Die beobachteten Ergebnisse reflektieren möglicherweise Konformations-Charakteristika, die von beiden Antikörpern unterschiedlich erkannt werden. Strukturelle Differenzen zwischen Vogel- und Säuger Antikörper mögen hierfür die Ursache sein. Korrelation der CCK-Gehalte aus beiden RIAs ergab eine signifikante Übereinstimmung. Da mit dem aviären Antikörper Verteilungsmuster von neuronalem CCK gefunden wurden, die von bisher Beschriebenem teilweise abweichen, werden die hier vorgestellten Befunde in diesem Zusammenhang diskutiert.

1 Introduction

CCK-8 belongs to a so-called brain-gut peptide family (Tatemoto 1983) which includes several molecular species (e.g. gastrin, CCK-58, CCK-33, CCK-4) having peripheral (nutrition) and central (neuronal transmission) biological functions. Antibodies raised against CCK usually cross-react with other CCK/gastrin members since these peptides share the last five amino acids and the antibodies are mostly directed against the C-terminal part of the molecule. Sequence-specific antibodies are rather scarcely available. Neuronal CCK was firstly described by Vanderhaeghen et al. (1975) and subsequently, largely identical distribution patterns of CCK in mammalian brain were described by several authors by using different antibodies (Loren et al., 1979; Innis et al., 1979; Larsson and Rehfeld 1979; Hökfelt et al., 1988; Seroogy et al., 1989 a,b; Pfister et al., 1989). Perikarya and fibres with CCK-like immunoreactivity (CCK-IR) were observed in several brain regions as e.g. substantia nigra (SN), ventral tegmental area (VTA), hippocampus, amygdala, cortex, whereas only fibres were found in the nucleus accumbens, striatum and tuberculum olfactorium. However, occasionally single somata with CCK-IR were observed in these brain regions (rats) (Takagi et al.,

1984; Hökfelt et al., 1988; Seroogy et al., 1989) but so far it is not defined under which experimental conditions these neurons have been visualised (perhaps it might be due to a pretreatment with colchicine). Recently, we have produced an antibody against CCK-8 in chickens which reproducibly stains perikarya in telencephalic rat brain regions (Schade et al., 1988; Pfister et al., 1989; Schade et al., 1991). Since these findings are at least partly in contrast to corresponding data from literature this study was performed to characterise the specificity of the avian antibody (IgY-antibody) in comparison to a rabbit antibody raised against the same conjugate.

2 Laboratory animals, material and methods

2.1 Animals

Chickens were housed in groups of five animals under SPF conditions in cages 2 × 2 m. The keeping parameters were in accordance with EG requirements. The experiment was performed in the „Agricultural Training- and Experimental-Station (Landwirtschaftliche Lehr- und Versuchsstation) of the Humboldt-University". From the point of animal welfare these housing conditions are not optimal, but there was no alternative at the time. The chickens were derived from the hybrid line „Medes white" (Deersheim, 13. inbred line). Rabbits (HsdPoc:NZW, breeding Harlan Winkelmann, Borchen) were kept in cages as usual. The animals were housed under a constant L:D (12:12 hours) regimen with food and water ad libitum. Chickens were fed with a standard food pellet with additional shell-lime and wheat grains to allow for a normal repertoire of behaviour. The rabbits likewise were fed with a standard food pellet with additional hay pellets to keep the rabbits busy.

The animals were immunised with an emulsion consisting of Freund's complete/incomplete (FCA/FIA, Difco) adjuvant and the antigen (CCK-8 Tyr SE coupled to BSA by means of glutaraldehyde, Sigma; CCK-8 Tyr SE is a modified CCK-sequence as shown in table 1). FCA was used as adjuvant because usually it is difficult to achieve an appropriate immune response against small coupled antigens like peptides. In chickens, the emulsion was injected i.m. in the *musculus pectoralis* whereas in rabbits an i.d. injection was used (multiple sites in the scapular region). A total of ca. 100 µg peptide was administered each time in a volume of max. 1 ml. Booster injections using FIA were performed at monthly intervals. The antibodies were extracted from egg yolk (chicken) as described previously (Schade et al., 1994) or sampled by bleeding (rabbit).

2.2 Antibody specificity

The peptides (table 1) used for testing specificity were synthesized according to usual procedures as described previously (Henklein et al., 1989). The peptides were compared on an equimolar base (peptide concentration in fMol at 50 % binding).

The specificity of the antibodies were studied using two different assays, a CCK-radio immunoassay (RIA) and a spot blot assay (SBA), respectively. A competitive radio immunoassay (RIA, Schade et al. 1988) was used to characterise the specificity of the antibodies with ^{125}I-CCK-8SE as tracer (Amersham). The rabbit antibody was diluted 1:100.000 (working dilution), the chicken antibody was utilised at a dilution of 1:1.000. The determination of CCK-content in rat brain samples was performed using both the „rabbit" and the „chicken" assay. The brain tissue samples originated from a study performed by Dr. K. Vick (Vick 1995).

The spot blot assay was performed using peptide-carrier (KLH) conjugates coupled with glutaraldehyde (CCK-8 SE, CCK-4) or with 1-ethyl-3,3-dime-

Table 1: Comparison of the log IC_{50} values obtained testing modified CCK-sequences in an „avian" and „mammalian" RIA, respectively.

Peptide	Sequence	log IC_{50} Ch	R
CCK-8SE	H- Asp-Tyr(SE)-Met-Gly-Trp-Met-Asp-Phe-NH_2	0,77	0,61
CCK-8NS	H-Asp-Tyr- Met-Gly-Trp-Met-Asp-Phe-NH_2	0,67	0,83
Succ-CCK-8SE	**Suc**-Tyr(SE)-Met-Gly-Trp-Met-Asp-Phe-NH_2	**0,80**	**0,47**
Turtle CCK-8SE	H-Asp-Tyr(SE)-**Pro**- Gly-Trp-Met-Asp-Phe-NH_2	1,05	1,80
Chicken CCK-8SE	H- **Phe**-Tyr(SE)-**Pro- Asp**-Trp-Met-Asp-Phe-NH_2	1,55	1,51
Gastrin 13SE	**...Glu- Ala-Tyr(SE)**- Gly-Trp-Met-Asp-Phe-NH_2	**0,52**	**1,36**
CCK-8 TyrSE	H- Asp-Tyr(SE)-Met-Gly-Trp-Met-Asp-Phe-**Tyr(SE)**-NH_2	**0,71**	**-0,61**
GE-410	**Suc**-Tyr(SE)-Met-Gly-Trp-Met-Asp-**PEA**	2,52	3,12
KI-1001	**Suc**-Tyr(SE)-Met-Gly-Trp-Met-**PEA**	6,29	6,29
CCK-5	H-Gly-Trp-Met-Asp-Phe-NH_2	**1,15**	**2,37**
Boc-CCK-5	**Boc**-Gly-Trp-Met-Asp-Phe-NH_2	**1,07**	**2,15**
CCK-5-Tyr	H-Gly-Trp-Met-Asp-Phe-**Tyr**-NH_2	5,61	6,29
CCK-4	H-Trp-Met-Asp-Phe-NH_2	2,46	2,96
CCK 2-6	**Suc**- Tyr-Met-Gly-Trp-Met-**OH**	—	—
Cycl. CCk-5	cycl(-**Asp**-Trp-Met-Asp-Phe-)	5,28	3,43
Cycl. CCK-8	cycl(-Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-)	3,45	3,45

The marked amino acids indicate the modifications compared to the original molecule. Marked IC_{50} values indicate major differences between them. Boc = tert. butyloxycarbonyl, PEA = ß-phenetylamid.

thylamino-carbodiimide (EDC, SuccC-CK-8 SE, KL 1001) according to usual coupling procedures. Aliquots of the freeze-dried conjugates were dissolved in 1µl DMSO and diluted with 0.1 M phosphate buffer (pH 7.4). Serial dilutions of the conjugate were prepared at constant KLH concentration (0. 25 mg/ml). Subsequently, 2 µl, containing 0.5µg – 0,005 µg of a conjugate, were spotted on nitrocellulose paper. As blanks, glutaraldehyde/EDC treated KLH were spotted. After blocking the protein binding capacity of the nitrocellulose paper with gelatin (0.1 %) and subsequent washing steps, the paper was incubated overnight with the rabbit anti-CCK antibody (1:1.200 diluted in Tris-buffered saline, TBS, pH 7.4) or with chicken anti-CCK antibodies (1:200 diluted in TBS). The primary antibodies were visualised by means of the PAP/DAB staining.

Additionally, by means of a sandwich enzyme immunoassay (EIA, Porstmann et al., 1988) the binding of anti-CCK antibodies from different chickens with coupled CCK in comparison with unchanged CCK (RIA) was studied. For this test, microplates (Nunc) were coated with an Ovalbumin-CCK-8 Tyr SE complex (3 µg/well). The antibody samples diluted to 1:100 with phosphate buffered saline (PBS, pH 7.2) were used.

3 Results

Table 1 shows the peptide sequences used for investigation of antibody-specificity and the corresponding IC_{50} values obtained using the „mammalian" or „avian" RIA. In fig. 1, a comparison is shown between several binding curves achieved with chicken or rabbit anti-CCK antibody. It can be seen that the avian antibody do not differentiate between the larger CCK-

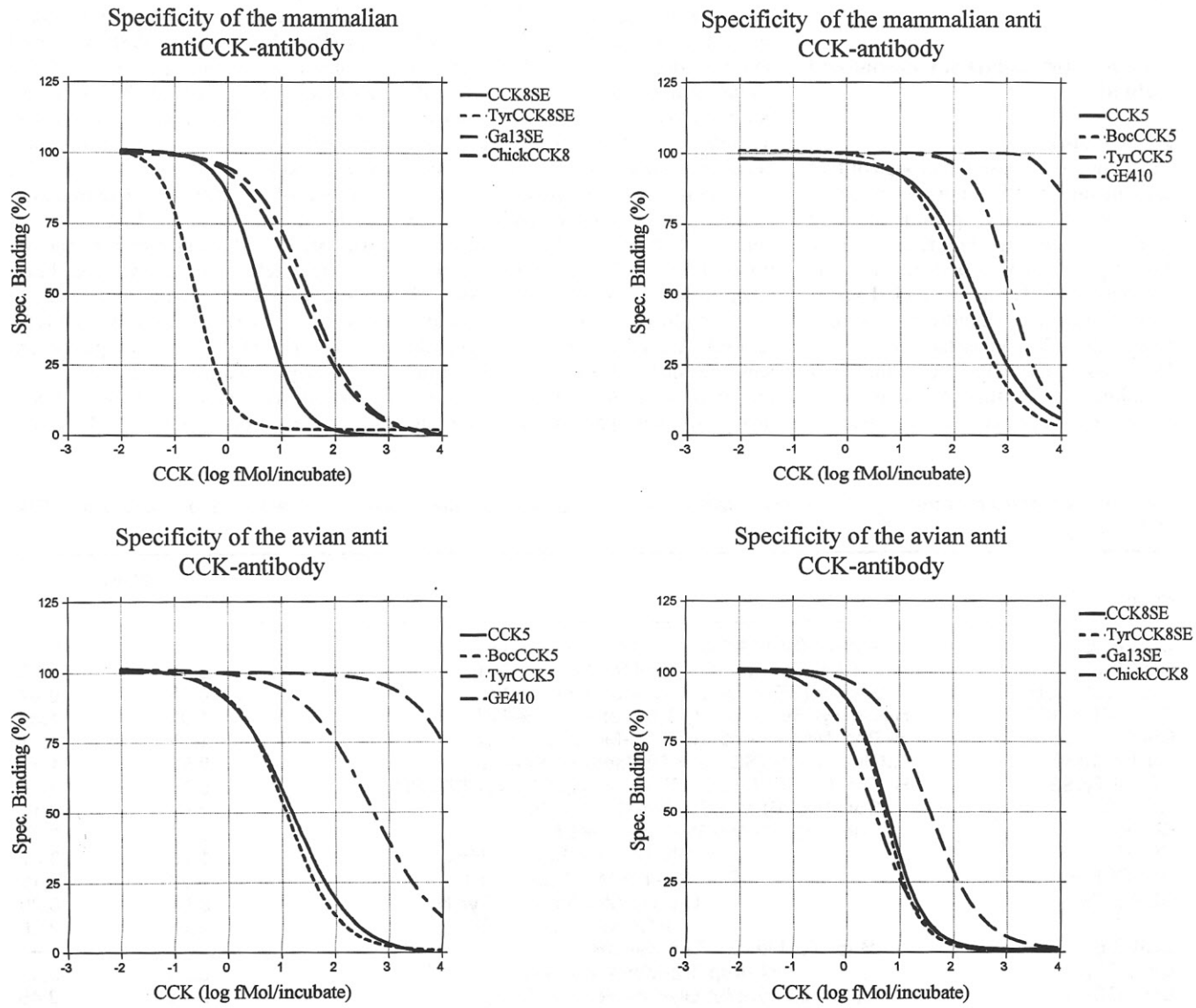


Figure 1: Specificity of the avian anti CCK-antibody in comparison to a corresponding mammalian antibody. Selected CCK-sequences are shown.

Table 2: Comparison of staining intensity obtained tested modified CCK-sequences by means of spot blot assay using avian and mammalian antibody, respectively

µg conjugate	CCK-8SE	SuccCCK-8SE	CCK-4	KI 1001	
0.5	+/-	-	+++	-	Chicken 89
0.05	-	-	-	-	
0.5	+	-	+++	-	Chicken 214
0.05	-	-	-	-	
0.5	+	-	+++	-	Chicken 158
0.05	+/-	-	+	-	
0.005	-	-	-	-	
0.5	+++	+/-	+++	+	Rabbit 4
0.05	+	-	+	-	
0.005	+	-	-	-	
0.0005	-	-	-	-	

The results are given as a semiquantitative evaluation of the staining intensity. As the coupling efficiency of the peptides to the KLH carrier protein is not known, the staining intensity is plotted against the amount of conjugate spotted on the nitrocellulose at constant carrier protein concentration. Chicken 89/rabbit 4 were immunised with CCK-8 SE whereas chicken 214 and 158 were immunised with CCK-8 TyrSE.

sequences (8 amino acids) in contrast to the mammalian antibody. Furthermore, there are partly remarkable differences between the corresponding IC_{50} values e.g. for gastrin and particularly for CCK-8 TyrSE. That holds also true for some of the short sequences as e.g. CCK-5 and BocCCK-5 which are more sensitively recognised by the avian antibody. This result is supported by data from the spot blot assay (table 2) which indicate a higher sensitivity of the IgY-antibody against CCK-4 in contrast to CCK-8 SE. However, a direct comparison between RIA-results and SBA-results is hardly possible since in the first case unchanged molecules are used in contrast to coupled molecules in SBA. These results strongly support the suggestion that the coupling procedure may change the molecule's conformation and consequently, leads to a changed binding behaviour (see the data for Succ-CCK 8SE). Fig. 2 shows a comparison between the binding activity of several avian anti-CCK-8 TyrSE antibody samples in RIA and EIA. As can be seen, there are only a few antibody samples working identically in both test systems. Most of the antibody-samples work either in RIA or in EIA which indicates that at least two different specificities exist.

The CCK content of rat brain tissue samples (striatum) was determined using an avian antibody in comparison to two different rabbit antibodies (raised against CCK-8 SE, antibody 4 and against CCK-8 Tyr SE, antibody 5). A statistical analysis yielded a significant correlation between the data obtained with the avian and the mammalian antibodies (fig. 3).

4 Discussion

The most crucial point in immunocytochemistry is the characterisation of specificity of the antibody used to visualise a certain substance. That is of particular importance, if tissue fixation is involved in staining procedures since the molecular structure of the substance of interest may be changed in non-defined ways. A simple control is the preabsorption of the antibody with the corresponding antigen. But this test may fail to work if the antibody is raised against a hapten-carrier conjugate whereas a „native" antigen is used for absorption. Therefore, several efforts were made to assess the specificity of antibodies by means of models which simulate the fixation procedure in vitro (Schipper and Tilders, 1983; Childs, 1983; Petrusz, 1983; De Vente et al., 1989; De Vente et al., 1993). In the present work, we used different test systems as well as coupled and non-coupled CCK-sequences to compare the specificity of avian with mammalian antibodies. The results obtained allow the following considerations:

In general, antibodies are able to recognise an amino acid sequence or a certain molecule conformation. From our data it can be seen that the C-terminal part of the molecule is necessary to be recognised by both the avian antibody and the mammalian antibody. Reduction of the C-terminal part leads to increased IC_{50}-values, while reduction of the N-terminal part or substitution of it had only marginal effects. However, extension of CCK-5 by Tyr resulted in a strongly increased IC_{50}-value in contrast to the non-modified CCK-5. Thus, N-terminal shortening and C-terminal extension (Tyr) together achieved a remarkable change in binding behaviour although the antibodies were raised against CCK-8TyrSE (see rabbit IC_{50} value). Comparing the IC_{50} values of the CCK-8 series with the values of CCK-5 and Boc-CCK-5, the differences are smaller in avian antibody in contrast to the mammalian antibody. Surprisingly, both antibodies were reactive with cyclic CCK. Based on these results it seems that conformational components of the molecules may influence the binding behaviour of the antibodies studied and, in addition, perhaps in a different way if IgG is compared with IgY. This assumption is supported by a study of Gerl et al. (page ©). These authors observed that a monoclonal mammalian antibody and a polyclonal avian antibody bound with the presumably same epitope of a human procollagen typ III molecule. However, only the avian antibody showed reactivity with both, rat and human derived procollagen. The assumption that conformational events may influence the binding behaviour is further supported by the results from SBA. In this assay the avian antibody preferentially reacts with KLH-coupled CCK-4 in contrast to the mammalian antibody.

A quantitative RIA for CCK content in striatal tissue of rat brain revealed a good correlation between the CCK-values obtained by the „avian" and the „mammalian" assays. There are two

Comparing of binding behaviour of different samples of avian anti CCK-antibodies in two different assays

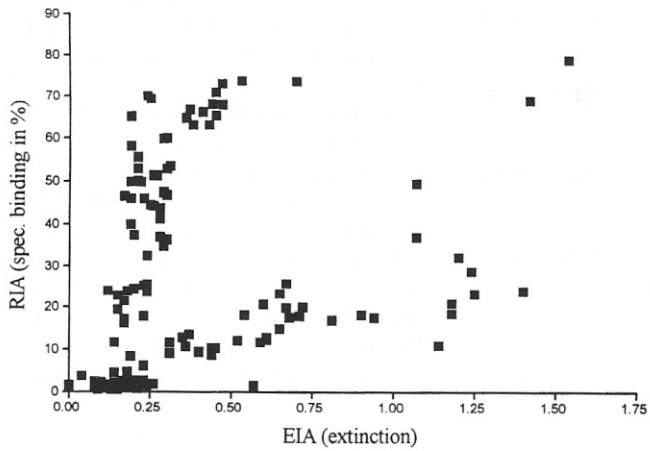


Figure 2: Shown is the binding of antibody-samples in a RIA in comparison to binding in EIA. Each point represents an antibody sample extracted from one egg. The eggs originated from a group of five hens kept together. Thus, the eggs could not identified and, consequently, a titre development could not registered.

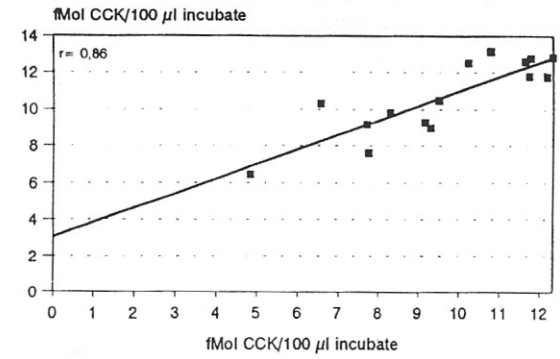
possibilities to discuss this result. 1.: It is thinkable, that the rabbit and chicken antibody each bind with several CCK-sequences but in a different ratio which finally results in a corresponding total CCK content. 2.: More simply, following the tissue preparation for CCK determination only one CCK-species is present in the probes to be measured. The differences between avian and mammalian antibody in recognising certain epitopes become important if the molecule is processed by fixation.

In conclusion, substantial differences between the binding behaviour of avian and mammalian anti CCK-antibodies in connection with the assay used have been observed. The reactivity of the avian antibody additionally seems to be influenced if coupled CCK-sequences are used instead of „native" molecules.

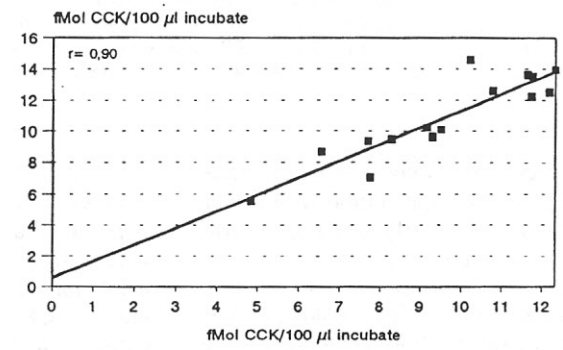
A prerequisite for the visualisation of neuronal CCK is the perfusion of the brain with a fixation solution. This procedure may result in a conformational change of neuronal CCK. Since CCK is synthesized in neurones as pre-pro peptide and subsequently processed during axonal transport, several CCK-species may be present in mammalian brain (Rehfeld, 1978). Thus, the different staining patterns obtained by using the avian antibody probably reflect the differenes in reactivity as

Striatal CCK content

Comparison of rabbit Ab 4 and Ab 5



Comparison of rabbit Ab 4 with chicken Ab



Comparison of rabbit Ab 5 with chicken Ab

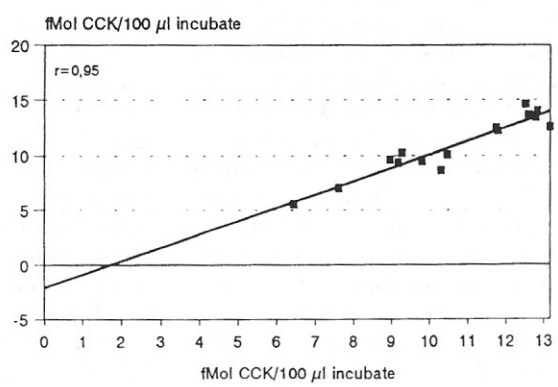


Figure 3: Shown is the comparison of striatal CCK-content measured by means of a RIA based on avian or mammalian antibody. Rabbit antibody 4 was raised against CCK-8SE, whereas rabbit antibody 5 was raised against CCK-8TyrSE. The statistical analysis revealed correlation coefficients of r = 0.86 (rabbit antibody 4/rabbit antibody 5), r = 0.90 (rabbit antibody 4/ chicken antibody), and r = 0.95 (rabbit antibody 5/chicken antibody).

discussed. In addition, there is a great phylogenetic distance between the mammalia and aves and structural characteristics of avian antibody differ significantly from mammalian antibody (Shimizu et al., 1992). Thus, the idea that a different reactivity of avian antibody may result in a different distribution pattern of neuronal CCK is in agreement with the results presented. Finally, the preparation of brain tissue for visualisation of small molecules like peptides causes conformational changes of many substances which may be recognised by avian but not by mammalian antibodies (and vice versa). Therefore, the introduction of avian antibodies in immunohistochemistry may be a valuable addition for studies on neuronal transmission.

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