Immunohistochemical localization of cocaine- and amphetamine-regulated transcript peptide in the brain of the pigeon (*Columba livia*) and zebra finch (*Taeniopygia guttata*)

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Introduction Cocaine- and amphetamine-regulated transcript (CART) is a relatively large peptide (ca. 100 amino acids) that acts as a neurotransmitter. In mammals, CART is heavily expressed in the striatum and the hypothalamus, but is also expressed in the retina, thalamus, cortex, cerebellum and several other regions. While the expression of CART has been thoroughly characterised in several mammalian species, it has only been shown in a few other vertebrate species and information about the expression of CART in reptiles and birds is completely lacking. Here, we show the distribution of the CART peptide (CARTp) using immunohistochemistry in the brains of the pigeon (*Columba livia*) and the zebra finch (*Taeniopygia guttata*).

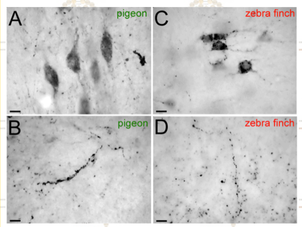

Figure 1: CART peptide immuno reactive (CARTp-ir) cells and terminals in the pigeon (**A-B**) and the zebra finch (**C-D**). CARTp-ir was found in the cytoplasm of cells, clearly labeling the cell body and main dendritic process Scale bars = 10 μm.

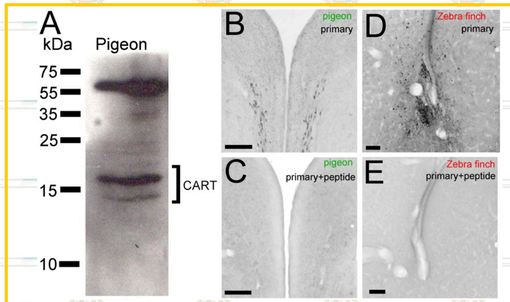

Figure 2. A. Western blot of pigeon brain homogenates probed with anti-CART peptide antibody. Two immunoreactive bands are detected around 15 kDA. **B** and **D** show labeling of CARTp-ir in neurons and terminals in both species. **C** and **E** show an equivalent section in which the primary antibody was pre-incubated with CART peptide. This removes labeling in both species. Scale bar =100 μm

Materials and Methods: Adult pigeons and zebra finches were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and either transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PFA, pH = 7.4) or their head removed and submerged in PFA. Brains were then removed and post-fixed for several days. Brains were cryoprotected in sucrose, embedded in gelatin and sectioned in the coronal plane at 40 μm. Serial sections through the brain were collected into several series (in 0.1M PBS) and immunoprocessed for CART using a rabbit anti-CART antibody (1:2000, CART (55-102), G-003-62, Phoenix Pharmaceuticals). The antibody was visualised using a biotinylated secondary antibody and a peroxidase-DAB reaction. Serial sections were viewed with a compound light microscope and images were acquired using a Retiga EXi FAST Cooled mono 12-bit camera and analyzed with OPENLAB imaging software. The images were compiled with PTGui v 6.0.3 and adjusted using Adobe Photoshop to compensate for brightness and contrast.

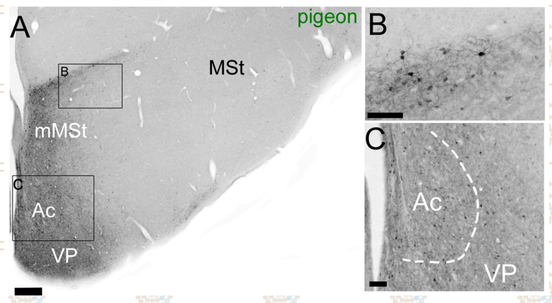

Figure 3: A CARTp-ir in the anterior part of the pigeon telencephalon. CART-ir cells and terminals were present along the medial subpallium, including the medial part of the medial striatum (mMST), the nucleus accumbens (Ac) and the ventral pallidum (VP). **B** CARTp-ir neurons in the border of mMSt and the nidopallium (N). **C** CARTp-ir cells and terminals through Ac and VP. Scale bars = 400 μm (A), 100 μm (B & C).

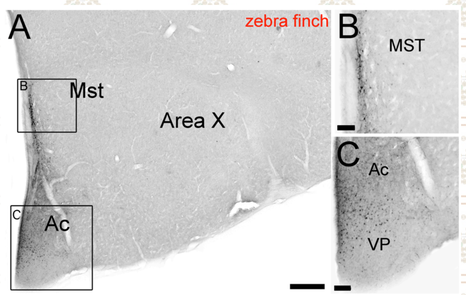

Figure 4: A CARTp-ir in the anterior telencephalon of the zebra finch. CARTp-ir neurons and terminals were foundalong the medial part of the subpallium, restricted to the medial most part of the MSt, Ac and VP. **B** show CART-ir neurons mMSt. **C** shows CARTp-ir neurons and terminals trough Ac and VP. Scale bars = 400 μm (A), 100 μm (B & C).

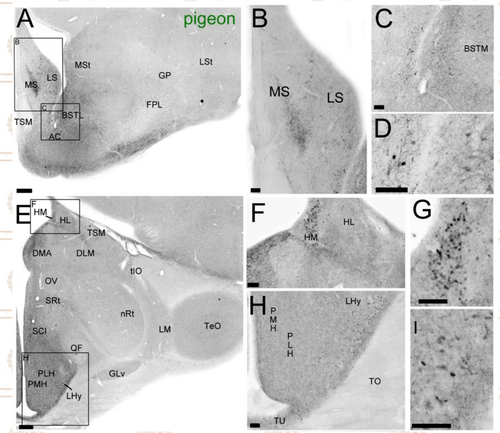

Figure 5: **A** CARTp-ir at the level of the septum. **B** Dense CARTp-ir terminals in the medial (MS) and lateral septal nuclei (LS). **C** CARTp-ir neurons in both sides of the ventral tip of the ventricle. In the medial side cells are strongly CARTp-ir while in the medial part of the bed nucleus of the stria terminalis (BSTM), are more weekly stained. **D** details of the CARTp-ir neurons shown in C. **E** CARTp-ir in the thalamus and hypothalamus of the pigeon. Dense CARTp-ir terminals occur in the ventral hypothalamus and along the midline, including the stratum cellulare internum (SCI) and the anterior dorsomedial thalamic nucleus (DMA). Weakly stained CARTp-ir neurons are found in nucleus sub rotundus (sRt) **F, G** CARTp-ir neurons in the medial habenular nucleus (HM). **H** dense CARTp-ir terminals in the ventral ventral hypothalamus. This includes the medial (PMH) and lateral (PLH) posterior hypothalamic nucleus and the lateral hypothalamic nucleus (Lhy). Scale bars = 400 μm (A & E), 100 μm (all others).

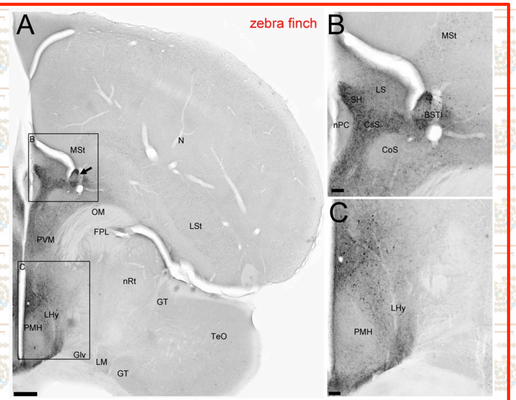

Figure 6: **A** Dense CARTp-ir terminals in the ventral hypothalamus and along the midline, including the magnocellular periventricular nucleus (PVM). **B** CARTp-ir terminals in the septum. Densely packed CARTp-ir terminals are found in the caudocentral septum (CcS) and the septohippocampal septum (SH). Less densely packed terminals are found in the ventral (LSc.v) and lateral (LSc.l) zones of the caudal division of the lateral septum and are almost absent from the dorsal zone (LSc.d), the commisural septal nucleus (CoS) and the nucleus of the pallial commisure (nPC). CART-ir cells and terminals also occur in the lateral part of the bed nucleus of the stria terminalis (BSTL). **C** CARTp-ir cells and terminals in the ventral hypothalamus. CARTp-ir terminals are absent or in low density in PMH, but are high in the regions around it. Strong CARTp-ir cells and terminals are found in the region immediately dorsal to PMH. Scale bar = 400 μm in A. 100 μm in B and C.

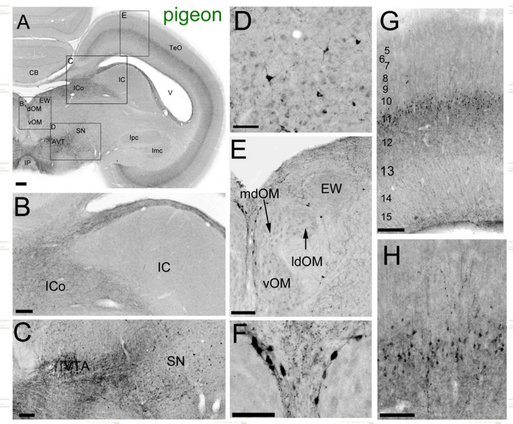

Figure 7: A. CARTp-ir in the pigeon at the level of the oculomotor nucleus. **B** large concentration of CARTp-ir terminals in the intercollicular (ICo) nucleus, but not in the inferior colliculus (IC). **C** CART-ir terminals in the ventral tecmental area (VTA) and substantia nigra (SN) and CARTir cells in the SN. **D** Details of the CARTp-ir cells in the SN. **E** a group of strongly CART-ir neurons medial and anterior to the oculomotor nucleus. **F** details of the CARTp-ir neurons in E. **G** CARTp-ir cells in layers 10-11 of the TeO. **H** show details of the CARTp-ir neurons showed in G and a CARTp-ir terminal. Scale bars = 400 μm (A), 200 μm (B,C&G), 100 μm (D,F&H).

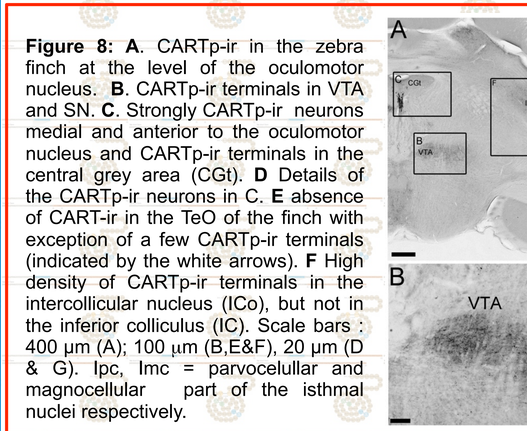

Figure 8: A. CARTp-ir in the zebra finch at the level of the oculomotor nucleus. **B**. CARTp-ir terminals in VTA and SN. **C**. Strongly CARTp-ir neurons medial and anterior to the oculomotor nucleus and CARTp-ir terminals in the central grey area (CGt). **D** Details of the CARTp-ir neurons in C. **E** absence of CART-ir in the TeO of the finch with exception of a few CARTp-ir terminals (indicated by the white arrows). **F** High density of CARTp-ir terminals in the intercollicular nucleus (ICo), but not in the inferior colliculus (IC). Scale bars : 400 μm (A); 100 μm (B,E&F), 20 μm (D & G). Ipc, Imc = parvocelullar and magnocellular part of the isthmal nuclei respectively.

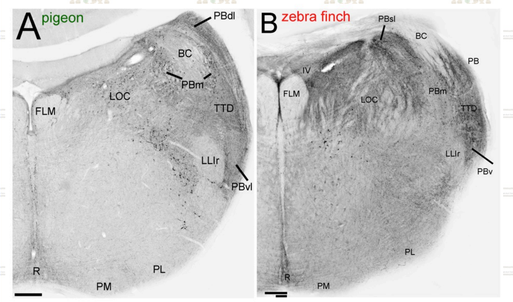

Figure 9: CARTp-ir in the brainstem of the pigeon (**A**) and Zebra finch (**B**). Dense CARTp-ir terminals aroundthe brachium conjunctivum (BC) in both species, in what correspond to the superior lateral (PBsl), medial (PBm) and ventral (PBv) parts of the parabrachial nucleus. Terminals are absent from the intermediate rostral lateral lemniscus nucleus (LLLIr) in both species. CART-ir terminals are also observed more medial, in the locus coeruleus (LOC) in both species and CARTp-ir cells in the pigeon. CARTp-ir terminal are also found in the raphe (R) nucleus. Scale bar = 400 μm.

Conclusions:

1. CARTp-ir occurs in neurons and terminals throughout the brains of both pigeons and zebra finches.

2. CARTp-ir is concentrated in subpallial regions, hypothalamus and structures associated with the limbic system in the mesencephalon and brainstem, with only a few differences between species.

3. Expression of CARTp peptide in cells and terminals in the limbic system and associated structures is highly conserved among vertebrates, but birds show little or no CARTp expression in the hippocampus, optic tectum, olfactory bulb and cerebellum.

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