Methodological details of histamine detection in the elongated portions of salivary gland cells by immune fluorescence in longitudinal cryosections (5 µm thickness) of the anterior segments of *Hirudo verbana*

A leech was moderately stretched out on a perforated wooden rod using pins and fixed in this position overnight at 4 °C in 4 % 1-ethyl-3(3-dimethylaminopropy)-carbodiimide (EDAC) in phosphate buffered saline (0.1 mol/l, pH 7.4). Post-fixation occurred using 4 % paraformaldehyde (PFA)-solution in PBS again overnight at 4 °C. The anterior segments (< 10) were cut off of the rest of the animal, transferred to a solution of 20 % (w/w) sucrose in PBS and incubated for 1 week at 4 °C. The sample was wrapped in aluminum wrap and frozen and stored at -84 °C.

The sample was placed on the freezing platform of a Leica CM1900 cryostat and embedded in Tissue Tek O.C.T (Sakura Finetek Europe B.V.) Frozen longitudinal sections (5 µm thickness) were prepared and transferred to microscopic slides. Sections were washed six times for 30 min in PBS and incubated overnight at 4 °C in a 1:1,000 dilution of primary antibody (anti-histamine, rabbit polyclonal, Cat No. 16043, PROGEN Biotechnik) in PBS-TX1 (100 ml PBS, 300 µl Triton-X-100, 1 g bovine serum albumin, 1 ml sodium azide (5 % stock solution)). Sections were washed four times for 30 min in PBS. The secondary anti-rabbit antibody was labeled with Alexa 488 (Invitrogen). Incubation occurred in a 1:50 dilution overnight at 4 °C. In addition, sections were counterstained using Hoechst nuclear dye (Cat No. 33342). Sections were washed six times for 30 min in PBS and coverslips were
mounted using Mowiol (Roth GmbH). Microscopic inspection and documentation was performed using a Leica TCS SP5 cLSM.

Histamine signals appear as yellowish regions, nuclei appear deep blue.