

SXN101742, a Botulinum Toxin-derived Targeted Secretion Inhibitor (TSI) inhibits GH secretion and synthesis: a new concept for the management of acromegaly



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Introduction & objectives

Botulinum neurotoxins (BoNTs) are zinc endopeptidases specifically blocking acetylcholine release in neuromuscular synapses. Molecular modifications of BoNTs prone to selectively target cells with aberrant secretion, also known as "Targeted Secretion Inhibitors" (TSIs), represent an emerging technology with promising therapeutic value. We designed and tested a new TSI (SXN101742) aimed at treating acromegaly, an endocrine disease due to a pituitary adenoma resulting in high GH and IGF-I levels. SXN101742, specifically targeted to somatotroph cells, involves a BoNT endopeptidase domain bearing a SNARE protein cleavage activity able to inhibit GH output from these cells. In order to provide evidence of the potential therapeutic benefit of SXN101742, we administered a single IV injection of this TSI to young rats, the growth of which naturally depends on a high GH/IGF-I activity, and followed different growth parameters over a 10-day period.

Materials & Methods

Construction, synthesis and purification of SXN101742/SXN101884
 Two versions were cloned: SXN101742, the active compound and SXN101884, an inactive control compound in which two codons were mutated within the endopeptidase encoding DNA rendering it catalytically inactive. Both proteins were expressed in an *E. coli* host before purification using a combination of affinity and classical chromatography methods to achieve a protein purity $\geq 95\%$, as assessed by SDS-PAGE densitometry, and an endotoxin level $< 5\text{EU/mg}$.

Animal protocols
 At 45 days of life (day0), Sprague Dawley rats were injected either with vehicle or a single bolus of:
 - SXN101742 at the dose of 1mg/kg (exp #1, pilot experiment)
 - SXN101742 at the incremental doses of 0.1, 0.3 and 1mg/kg (exp #2, dose-response study)
 - SXN101742 or SXN101884 (endopeptidase-inactivated molecule) at the dose of 1mg/kg (exp #3, endonegative experiment).
 Body weight and food intake were monitored daily. Blood samples were collected on days 0 (before the injection) and on days 3, 6 and 9 after the injection. On day 10, rats were anesthetized and measured (nose to anus length) before sacrifice. Pituitaries and others organs were weighed before being fixed in paraformaldehyde (PAF) or frozen in liquid nitrogen and stored at -80°C until later analyses.

Histology
 Pituitary glands were fixed in PAF for 2h before being dehydrated and embedded in paraffin. 5 μm sections were stained with hematoxylin and eosin (H&E) or incubated with primary anti-GH antibody (Dr Parlow, Torrance, CA, USA) and secondary Alexa Fluor® 555 conjugated IgG fluorescent antibody (Invitrogen, Switzerland).

GH and IGF-I measurements
 Plasma GH levels and pituitary contents were measured using an Elisa kit EZRMGH-45K (Millipore, USA). Plasma IGF-I levels and hepatic content were measured using an Immunoenzymetric assay kit IEMA AC-18F1 (Immunodiagnosics Systems, UK).

Gene expression analysis
 Total RNA from pituitaries or liver were extracted using the RNeasy Mini Kit® (Qiagen, Switzerland). Two micrograms total RNA were reverse-transcribed using Moloney Murine Leukemia Virus Reverse Transcriptase kit (Invitrogen, Switzerland). Real-time PCR were performed using a Master SYBR Green mix and an ABI StepOne Plus Detection System (Applied Biosystems, Switzerland). Results were normalized using the housekeeping gene RPS29 and tubulin and expressed in arbitrary units (A.U.).

Bone development analysis
 Length and cross sectional area of the midshaft femur, as well as the trabecular bone architecture of the distal femur were assessed using a MicroCT UCT40 (Scanco Medical AG, Switzerland). Mineral apposition rate and growth plate thickness were determined by histomorphometry using respectively the calcein double labelling method and the toluidine blue staining.

Statistics
 Results are expressed as mean \pm SEM for the indicated number of observations. The unpaired Student's t-test and repeated-measures analysis of variance (ANOVA) were used when appropriate for comparison between groups. A p value of < 0.05 was considered statistically significant.

Conclusions

A single bolus of SXN101742 to juvenile rats produced an inhibition of both GH secretion and synthesis with downstream major inhibitory effects on growth parameters, making it a valuable new candidate to treat acromegaly. " Targeted Secretion Inhibitors " represent a new class of molecules for the treatment of excessive hormonal secretion.

Results (exp #1)

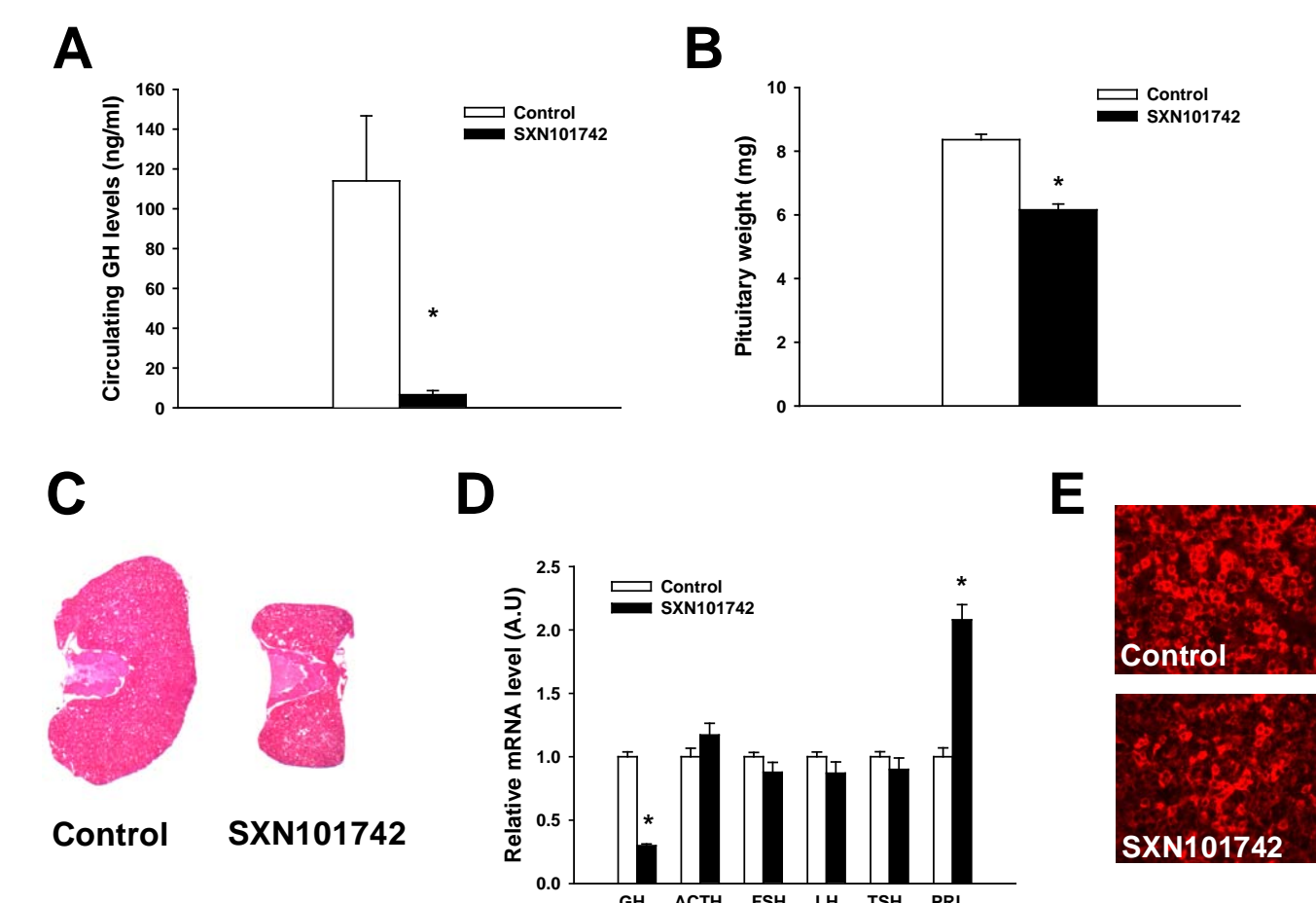


Fig.1: Circulating GH levels and pituitary gland characteristics of SXN101742-treated growing rats (Exp. #1)
 A: Plasma GH levels (ng per ml).
 B: Total weight of pituitary glands (mg).
 C: Hematoxylin and Eosin staining of pituitary sections.
 D: Relative gene expression for the anterior pituitary hormones (Arbitrary Units).
 E: GH immunostaining.
 For all panels, 45 day-old male rats received either a single i.v. injection of SXN101742 at the dose of 1 mg/kg (■) or vehicle (□) and were studied 10 days later. Results are expressed as means \pm SEM of N=10-12 animals per group. Student's t-test: *p < 0.005 vs. control group for all panels.

Results (exp #2)

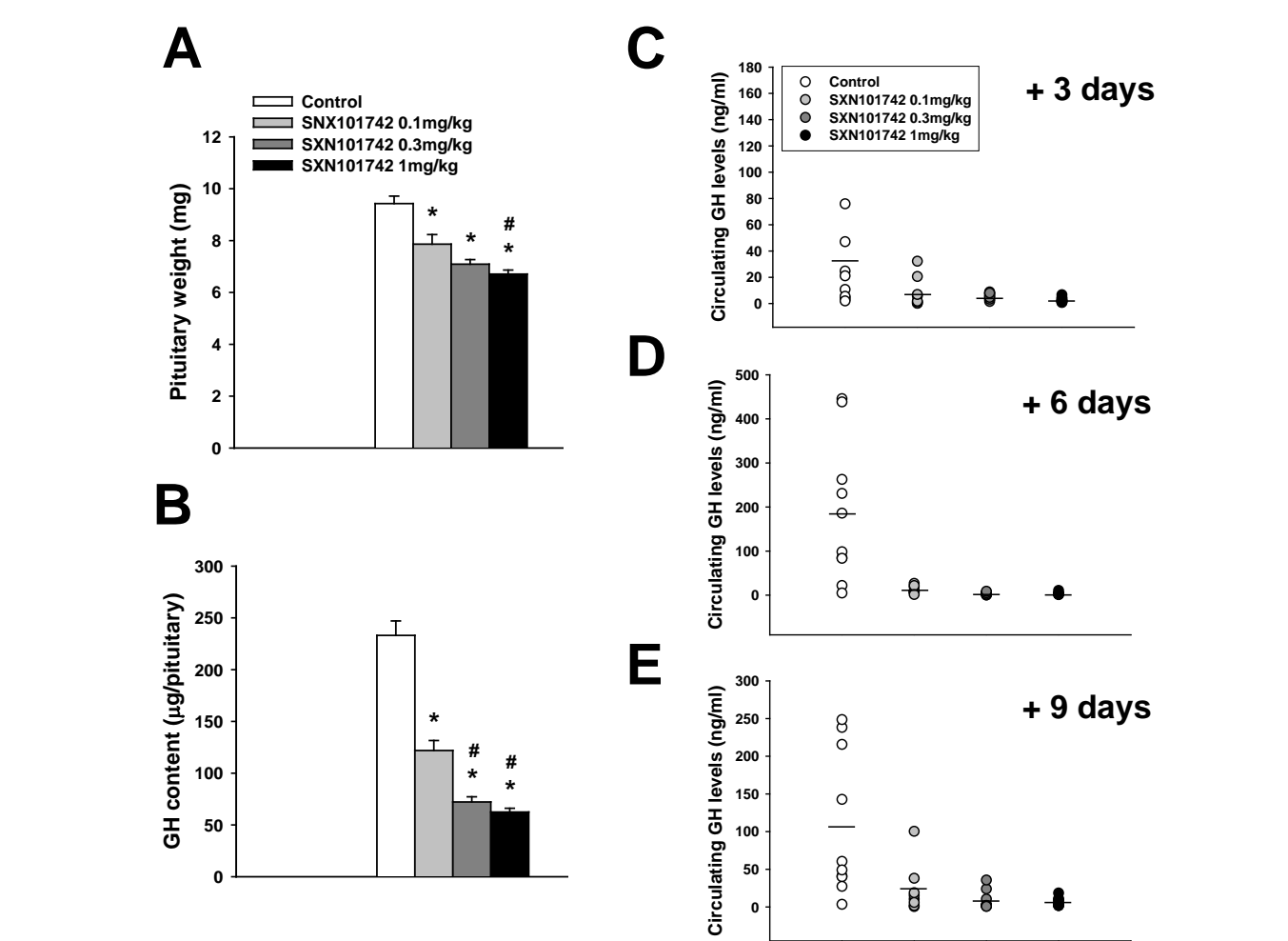


Fig.3: Dose-response effect of SXN101742 on GH production and secretion in growing rats (Exp. #2)
 A: Total weight of pituitary glands (mg). *p < 0.005 vs. control group and *p < 0.01 vs. 0.1 mg/kg group.
 B: GH content of pituitary glands (μg). *p < 0.001 vs. control group and *p < 0.001 vs. 0.1 mg/kg group.
 C, D and E: Plasma GH individual levels (ng per ml), 3 days (panel C), 6 days (panel D), and 9 days (panel E) after treatment.
 For all panels, 45 day-old male rats received either a single i.v. injection of SXN101742 at the dose of 0.1 mg/kg, 0.3 mg/kg, 1 mg/kg or vehicle and were studied 10 days later or at the indicated time point. Results are expressed as means \pm SEM of N=8-10 animals per group.

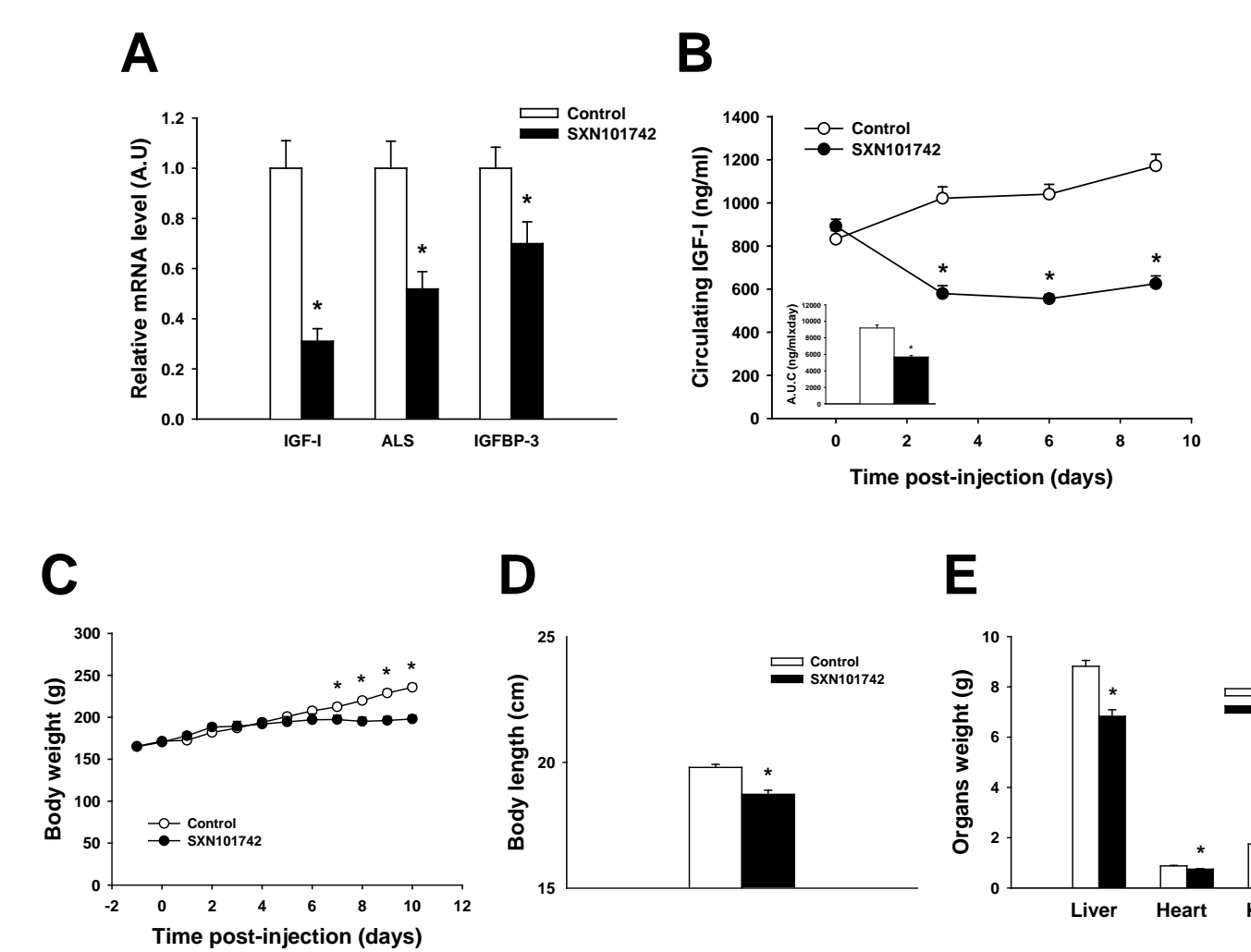


Fig.2: IGF-I levels and somatic growth of SXN101742-treated growing rats (Exp. #1)
 A: Relative gene expression for IGF-I, ALS and IGFBP-3 in liver (Arbitrary Units). *p < 0.001 for IGF-I, *p < 0.002 for ALS, *p < 0.05 for IGFBP-3 vs. respective control group.
 B: Time course of plasma IGF-I levels (ng per ml). *p < 0.001 vs. control group. Insert panel: area under the curve (A.U.C), *p < 0.001 vs. control group.
 C: Body weight curves. *p < 0.02 vs. control group.
 D: Body length (cm). *p < 0.001 vs. control group.
 E: Weight of organs (liver, heart, kidney) (g). *p < 0.001 vs. control group.
 For all panels, 45 day-old male rats received either a single i.v. injection of SXN101742 at the dose of 1 mg/kg (■) or vehicle (□) and were studied 10 days later. Results are expressed as means \pm SEM of N=12 animals per group.

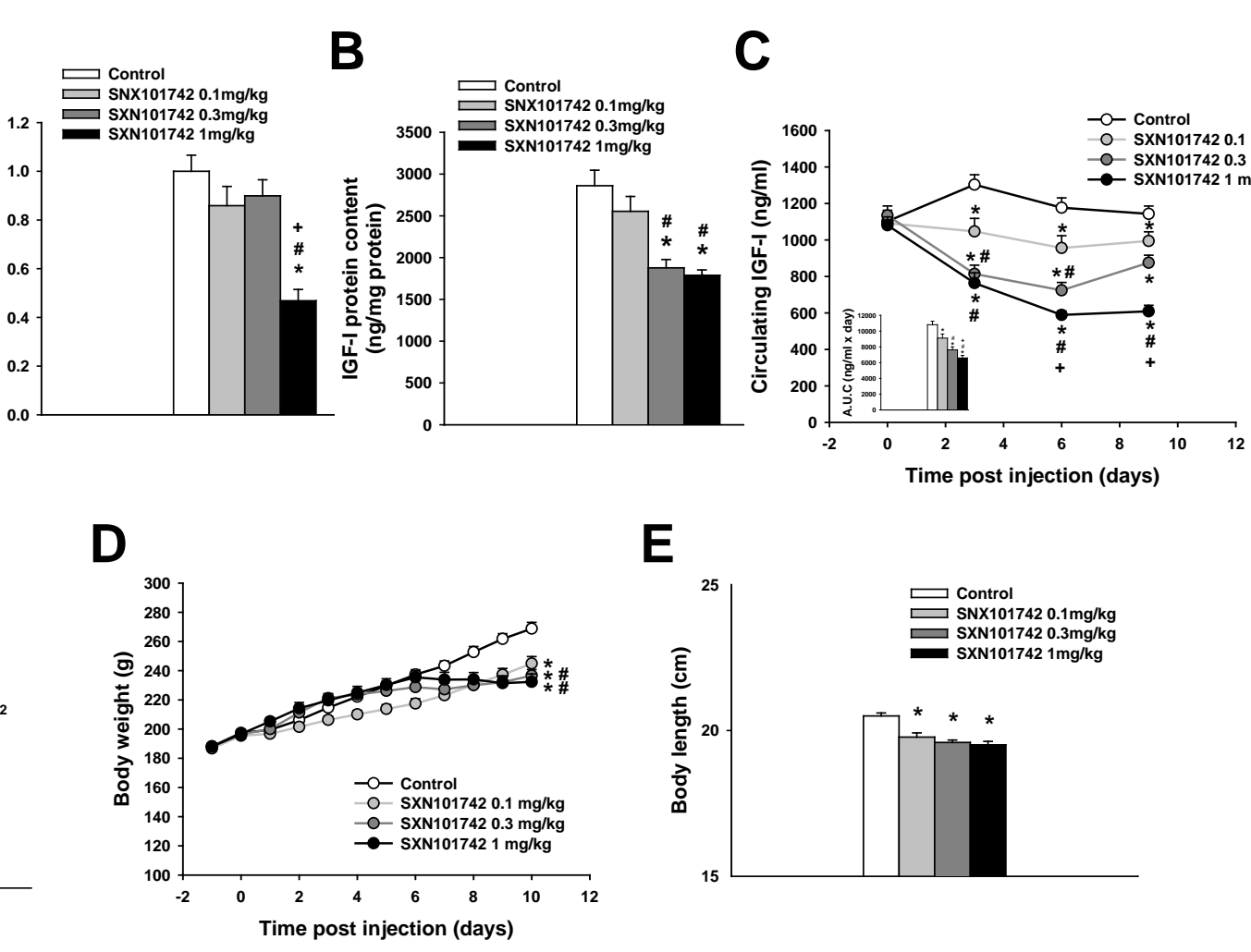


Fig.4: Dose-response effect of SXN101742 on IGF-I secretion and somatic growth in growing rats (Exp. #2)
 A: Relative gene expression for IGF-I in liver (Arbitrary Units). *p < 0.001 vs. control group, *p < 0.001 vs. 0.3 mg/kg group.
 B: IGF-I protein content in liver (ng per mg of protein). *p < 0.001 vs. control group, and *p < 0.01 vs. 0.1 mg/kg group.
 C: Time course of plasma IGF-I levels (ng per ml). *p < 0.05 vs. control group, *p < 0.05 vs. 0.1 mg/kg group. *p < 0.05 vs. 0.3 mg/kg group. Insert panel: area under the curve (A.U.C), same significance as in the main panel.
 D: Body weight curves. *p < 0.05 vs. control group, *p < 0.05 vs. 0.1 mg/kg group.
 E: Body length (cm). *p < 0.001 vs. control group.
 For all panels, 45 day-old male rats received either a single i.v. injection of SXN101742 at the dose of 0.1 mg/kg, 0.3 mg/kg, 1 mg/kg or vehicle and were studied 10 days later or at the indicated time point. Results are expressed as means \pm SEM of N=8-10 animals per group.

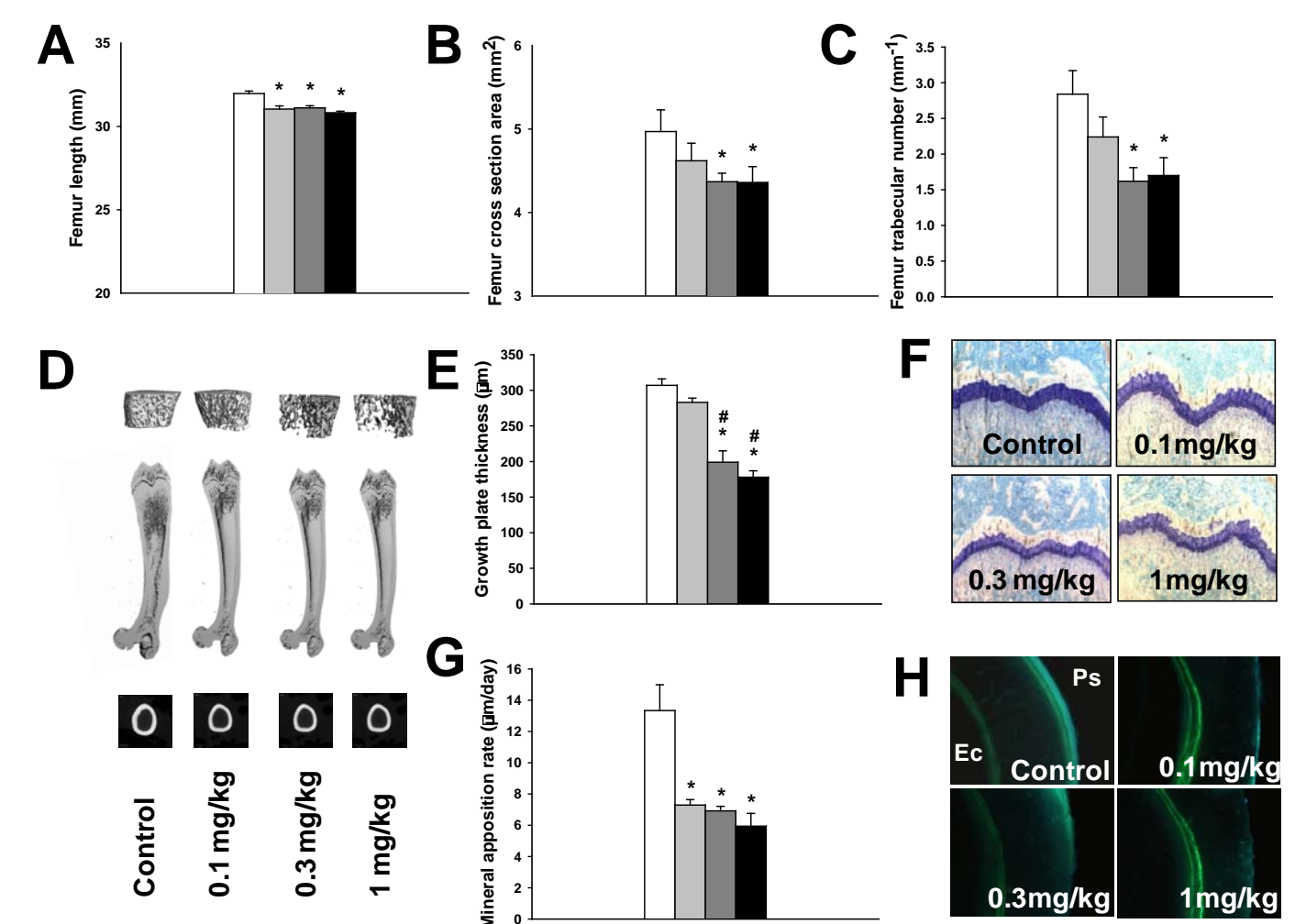


Fig.5: Dose-response effect of SXN101742 on bone mass acquisition in growing rats (Exp. #2)
 A: Femur length (mm). *p < 0.001 vs. control group.
 B: Femur cross section area (mm²). *p < 0.05 vs. control group.
 C: Femur trabecular number (mm⁻³). *p < 0.01 vs. control group.
 D: Images of tridimensional reconstruction of distal trabecular structures (top panel), longitudinal sections (middle panel) and transversal sections (bottom panel) of femurs.
 E: Growth plate thickness (μm). *p < 0.001 vs. control group and *p < 0.01 vs. 0.1 mg/kg group.
 F: Radial growth histology. Growth plates were stained using a toluidine blue staining.
 G: Mineral apposition rate ($\mu\text{m/day}$). *p < 0.05 vs. control group.
 H: Radial growth histology. Femur sections were stained using the calcein double labelling method. Ps: periosteal surface, Ec: endocortical compartment.
 For all panels, 45 day-old male rats received either a single i.v. injection of SXN101742 at the dose of 0.1 mg/kg, 0.3 mg/kg, 1 mg/kg or vehicle and were studied 10 days later. Results are expressed as means \pm SEM of N=8-10 animals per group for panels A, B and C or N=5 animals per group for panels E and G.

Results (exp #3)

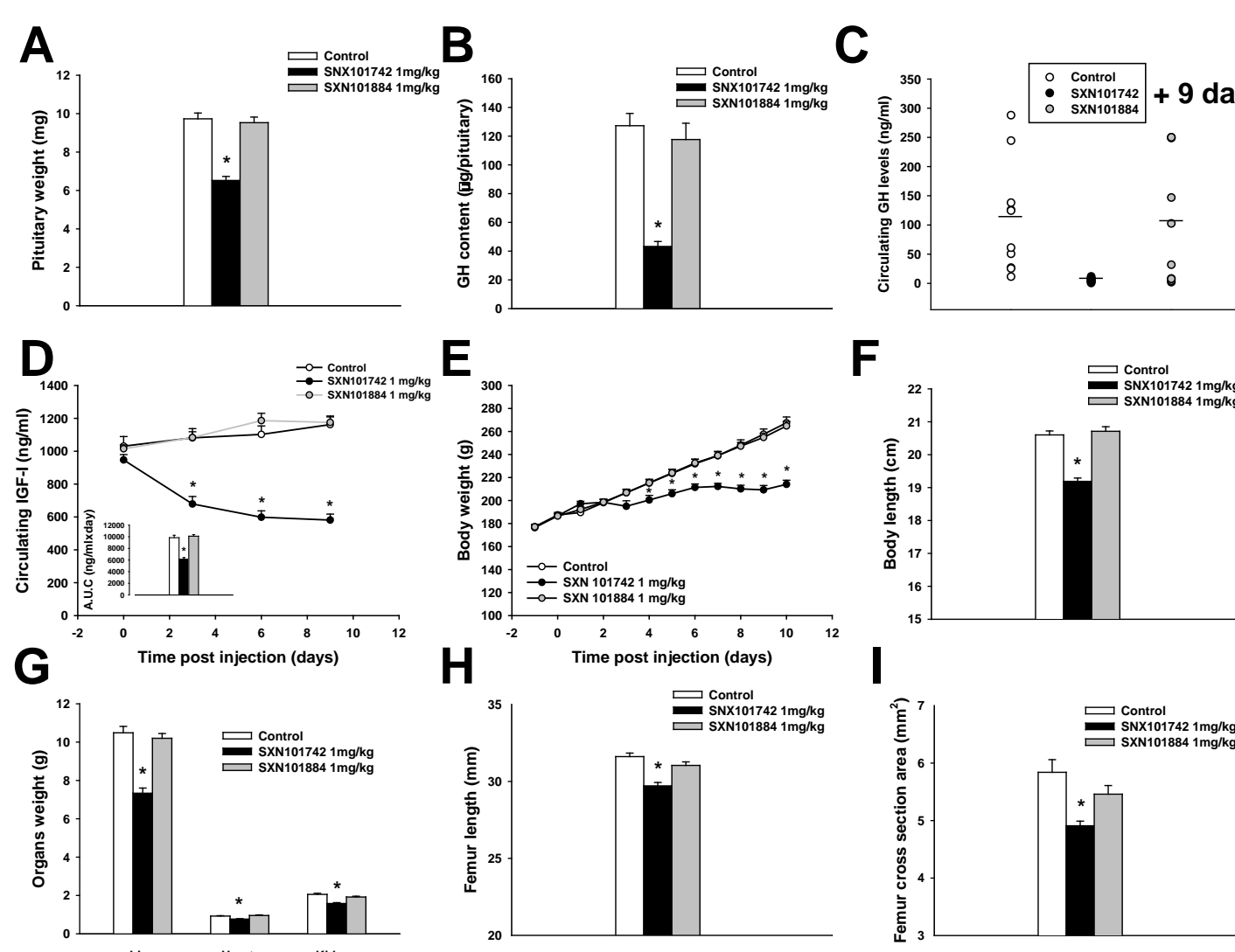


Fig.6: Inactivation of endopeptidase activity abolishes GH/IGF-I inhibitory function in SXN101884-treated growing rats (Exp. #3)
 A: Total weight of pituitary glands (mg). B: GH content of pituitary glands (μg). C: Plasma GH individual levels (ng per ml) 9 days after treatment. D: Time course of plasma IGF-I levels (ng per ml). E: Body weight curves. F: Body length (cm). G: Weight of organs (g). H: Femur length (mm). I: Femur cross section area (mm²).
 For all panels, 45 day-old male rats received either a single i.v. injection of SXN101742 or SXN101884 at the dose of 1 mg/kg or vehicle and were studied 10 days later or at the indicated time point. Results are expressed as means \pm SEM of N=10 animals per group.