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Dual Role of Insulin in Spexin Regulation : Functional Link between Food Intake and Spexin Expression in Fish Model

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Abbreviations: SPX, Spexin; IGF-I/-II, Insulin-like growth factor-I/-II; InsR, Insulin receptor; IGF1R, IGF1 receptor; MAPK, Mitogen-activated protein kinases; PI3K, Phosphoinositide 3-kinase; Akt, Protein kinase B; $MEK_{1/2}$, Mitogen-activated protein kinase kinase 1/2; $ERK_{1/2}$, Extracellular signal-regulated kinase 1/2.

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Abstract

Spexin, a neuropeptide discovered by bioinformatics approach, has been recently identified as a satiety factor in fish model. However, the functional link between feeding and spexin expression as well as the signal transduction for spexin regulation is totally unknown. Here we used goldfish as a model to examine the functional role of insulin as a postprandial signal for spexin regulation in bony fish. In goldfish, feeding could elevate plasma levels of glucose, insulin and spexin with concurrent rises in insulin and spexin mRNA expression in the liver. Similar elevation in spexin mRNA level was also observed in the liver and brain areas involved in appetite control in goldfish after intraperitoneal injection of glucose and insulin, respectively. By parallel experiments with goldfish hepatocytes and brain cell culture, insulin signal induced by glucose was shown to exert dual role in spexin regulation, namely (i) acting as autocrine/paracrine signal to trigger spexin mRNA expression in the liver, and (ii) serving as endocrine signal to induce spexin gene expression in the brain. Apparently, the peripheral (in the liver) and central actions of insulin (in the brain) on spexin gene expression were mediated by insulin receptor (to lesser extent by IGF-I receptor) coupled to $MKK_{3/6}/P_{38}$ ^{MAPK} and PI3K/Akt/mTOR but not $MEK_{1/2}/ERK_{1/2}$ cascades. Our findings indicate that an insulin component inducible by glucose is present in the liver of fish model and may serve as the postprandial signal linking food intake with spexin expression both in the central as well as at the hepatic level.

(249 words)

Introduction

Spexin (SPX), the protein product of C12orf39 gene in human, is a novel peptide first identified by bioinformatics prior to its purification/functional studies (1, 2). It is a 14-amino acid (a.a.) peptide with C-terminal α-amidation and its protein sequence is highly conserved from fish to mammals (3-5). In representative species, e.g., human (6), rat (7), mouse (2), and more recently in fish models (3-5, 8), SPX is widely expressed at tissue level suggesting that the peptide may have pleiotropic functions. This idea is consistent with the findings that SPX is involved/implicated in gastrointestinal motility (1, 9), feeding and energy balance (4, 5), luteinizing hormone release (8), adrenocortical cell proliferation (10), nociception and cardiovascular/renal function (11) as well as O_2 sensing in the carotid body (12). In human, low levels of serum SPX can be associated with insulin and lipid resistance (13), especially for type II diabetes (6) and both adult (14) and childhood obesity (15), which has aroused the interest in developing SPX-based analogs with pharmacological/therapeutic potential (16). Recently, SPX has been shown to activate galanin type II and III receptors expressed in HEK293 cells (17), suggesting that the two receptors may act as the cognate receptors for SPX. Despite the recent progress on SPX research, there is still a lack of information regarding the endocrine control of SPX expression and the signal transduction mechanism for SPX regulation is totally unknown.

To shed light on the comparative aspects of SPX in lower vertebrates, especially in fish species, we used goldfish as a model to establish the solution structure of fish SPX by NMR and confirm its expression at protein level within the brain by LC/MS/MS (4). Using a combination of in vivo and in vitro approaches, SPX expression in brain areas involved in appetite control was shown to be elevated after food intake and SPX treatment via differential regulation of orexigenic and anorexigenic signals within these brain areas (by increasing CART, CCK & POMC with concurrent drop in NPY & AgRP expression) could inhibit feeding behavior and food consumption in goldfish (4), indicating that SPX can act a novel satiety factor in fish model. Recently, the association of SPX expression with feeding has also been reported in fish species with commercial value, e.g., grouper (3) and Ya-fish (5), which raise the possibility of using SPX as a new target for manipulating/improving body growth and energy

balance in cultured fish. Although our study in goldfish has clearly demonstrated a central component of SPX expression in appetite control, the possibility for peripheral input of SPX was not examined. Furthermore, the functional link between food intake and SPX expression as well as the underlying mechanisms for SPX regulation are still unclear and remain to be investigated.

Since insulin is a major component of glucose homeostasis after feeding, and by itself, also acts as a satiety factor in mammals through modulation of orixegenic/anorexigenic signals (18), it raises the possibility that insulin may play a role in SPX regulation. In this study, we sought to test the hypothesis that glucose-induced insulin release/production caused by food intake might serve as a postprandial signal linking feeding with SPX expression in goldfish. Whole animal experiments were conducted to investigate the effects of (i) food intake and (ii) intraperitoneal (IP) injection of glucose and insulin, respectively, on SPX expression in the liver and/or brain areas known to be involved in appetite control in goldfish. In vitro studies were also performed in goldfish hepatocytes and brain cell culture to elucidate the receptor specificity as well as post-receptor signaling for SPX regulation by insulin using a combination of pharmacological approach and direct monitoring of activation status for signaling targets. To our knowledge, our study represents the first report to (i) provide evidence for the dual role of insulin as both the endocrine as well as autocrine/paracrine signal triggered by feeding for SPX regulation, and (ii) establish the signal transduction mechanisms for SPX expression both in the central (the brain) as well as at the peripheral level (the liver).

Materials and Methods

Animal and test substances

Goldfish (*Carassius auratus*) with 20-35 g body weight were purchased from local pet stores and maintained in well-aerated 650 L aquaria at 20 ^{o}C under 12D:12L photoperiod for more than 14 days prior to in vivo experiments/harvesting tissue for cell culture. Since the fish used in our study were

sexually regressed and sexual dimorphism was not apparent, mixed sexes of goldfish were used for in vivo and in vitro experiments. Drug treatment and tissue sampling were conducted as described by the protocols (CULATR#3890) approved by the Committee for Animal Use in Teaching and Research at the University of Hong Kong. Human insulin and its antagonist S961 was obtained from Sigma (St. Louis, MO) while the inhibitors for insulin receptor (InsR) and/or IGF-I receptor (IGF1R), including picropodophyllin (PPP), 2-hydroxynaphthalenyl-methylphosphonic acid (HNMPA) and N-(5-chloro-2-methoxyphenyl)-N'-(2-methyl-4-quinolinyl)urea (PQ401), and blockers for target signaling kinases, including 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene (U0126), 2-(2-amino-3-methoxyphenyl)4H-1-benzopyranone (PD98059), 5-(2-phenylpyrazolo[1,5-a]pyridin-3-yl)-1H-pyrazolo-[3,4-c]pyridazin-3-amine (FR180204), (3R)-1-[2-oxo-2-[4-[4-(2-pyrimidinyl)phenyl]-1-piperazinyl]-ethyl]-N-[3-(4-pyridinyl)-1H-indazol-5-yl]-3-pyrrolidinecarboxamide (SCH772984), 4-(4-fluorophenyl)-2-(4-nitrophenyl)-5-(4-pyridyl)-1H-imidazole (PD169316), 4-[5-(4-fluorophenyl)-2-[4-(methylsulfonyl)phenyl]-1H-imidazolyl]-pyridine (SB203580), wortmannin, 2-(4-morpholinyl)-8-phenyl-4H-1-benxopyrin-4-one (LY294002), triciribine hydrate (API-2) and rapamycin, were acquired from Calbiochem (San Diego, CA). These test substances were dissolved in PBS or DMSO, stored frozen in small aliquots at -80 ºC and diluted with prewarmed culture medium to appropriate concentrations 10 min prior to drug treatment.

In vivo experiments for postprandial effect of feeding and glucose/insulin treatment

For in vivo experiments, goldfish (8-10 fish/group) were acclimated for ≥14 days to single housing in 25-liter tanks with a one-meal-per-day feeding schedule (with daily food supply at 10:00 AM). To study the post-prandial effects of feeding, the fish were divided into two groups, one with regular supply of food pellets (~1% BW, as "fed group") while the other without food provision (as "unfed group"), and the time for daily administration of fish feed was taken as time zero (0 hr) for reference. For in vivo treatment with glucose and insulin, IP injection of D-(+)-glucose or human insulin dissolved in fish physiological saline were conducted in goldfish under MS222 anesthesia 5 min prior to the regular feeding time but with no food provision after the fish recovered from the anesthesia

(which took 3-5 min for full recovery). Sampling of blood and tissues was performed according to standard procedures (4) at the time points as indicated. Circulating levels of glucose, insulin and SPX were measured using Glucose LiquiColor test (Stanbio Laboratory, Texas, USA), Fish Insulin ELISA kit (Cusabio Life Sci, Wuhan, China) and SPX Fluorescent EIA kit (Phoenix Peptides, Arizona, USA), respectively. Total mRNA isolation, RT sample preparation and subsequent real-time PCR for target genes were carried out as described previously (19) with qPCR conditions listed in Supplemental Table (1). Authenticity of PCR products were confirmed by melting curve analysis and parallel measurement of 18S RNA was used as the internal control. To evaluate insulin expression at tissue level and InsR and IGF1R expression both in the hepatocytes and brain cell culture, RT-PCR was also performed for tissue expression profiling of insulin as well as detection of multiple isoforms of InsR/ IGF1R expressed in the respective cell culture with PCR protocols described in Supplemental Table (2). In these studies, RT-PCR for β-actin was used as the quality control for RNA preparation.

In vitro experiments with goldfish hepatocytes and brain cell culture

Goldfish hepatocytes (with viability ≥94%) were prepared by collagenase digestion method with minor modifications (19) and cultured in serum-free DMEM/F-12 medium (Invitrogen) at 0.8×10^6 cells/ml/well in 24-well plates. Goldfish brain cells (with viability ≥92%) were prepared from brain areas covering the telencephalon, optic tectum and hypothalamus by trypsin digestion as described previously (4) and cultured in 35-mm dishes at 3×10^6 cells/2 ml/dish with Neurobasal (Invitrogen) containing B27 supplement (1:50 dilution). Cell cultures for the two target tissues were routinely prepared from tissues harvested from 6 goldfish and maintained at 28 ºC under 5% CO_2 and saturated humidity for 15 hr for carp hepatocytes (to avoid cell aggregation caused by prolonged culture) and 7 days for brain cells (to allow for neuron recovery & glial cell attachment) prior to initiation of drug treatment. After drug treatment, total RNA was extracted and subjected to real-time PCR for target gene measurement as described in the preceding section. Real-time PCR for 18S RNA and β-actin were also conducted to serve as the internal control for hepatocyte and brain cell culture, respectively. For in vitro studies on SPX secretion, goldfish hepatocytes were cultured at 15×10^6 cells/2 ml/dish in

60 mm dish and exposed to drug treatment in the presence of 1 × dilution of the Complete ™ protease inhibitor cocktail (Roche). After that, culture medium harvested was concentrated using C18 Sep-Pak chromatography (Waters) and used for SPX measurement with SPX Fluorescent EIA kit.

Western blot of kinase activation induced by insulin treatment

To examine the post-receptor signaling for insulin-induced SPX gene expression, Western blot was performed in cell lysate prepared from goldfish hepatocytes or brain cell culture after insulin treatment as described previously (20) using antibodies specific for the phosphorylated form ("P-") and total protein ("T-") of $MEK_{1/2}$ (1:1,000), $ERK_{1/2}$ (1:5,000), $MKK_{3/6}$ (1: 2,000), $P_{38}{}^{MAPK}$ (1: 1,000), PI3K (1:2,000) and Akt (1:1,500), respectively (see Antibody Table for the details of these antibodies and their respective suppliers). In these studies, parallel blotting of β-actin was used as the loading control. Quantitation of the results of Western blot was performed by densitometric scanning using ImageJ (https://imagej.nih.gov/ij/) and the data was transformed as a ratio of the phosphorylated form over the total protein of the respective targets.

Data transformation and statistics

For real-time PCR, standard curves with dynamic range of $\geq 10^5$ and correlation coefficient of $\geq$ 0.95 were constructed for data calibration with serial dilutions of plasmids carrying the ORF of target genes. The raw data of mRNA expression for in vivo experiments (in terms of femtomole transcript detected/assay tube) were normalized against 18S RNA expression detected in the same sample and transformed as a percentage of the mean value for target gene expression in control group at time zero (i.e., as "%Ctrl" of the reference group at 0 hr). For in vitro experiments with cell culture, the raw data of mRNA expression were measured in terms of femtomole transcript/10^6 cells/well. These data were normalized against β-actin mRNA in the case of brain cell culture and transformed as a percentage of the control group without drug treatment (as "%Ctrl"). Since 18S RNA levels (the internal control for hepatocytes) did not exhibit notable changes in our in vitro experiments, the raw data for target gene

192 expression in goldfish hepatocytes were simply transformed as a percentage of the mean value in the
193 control (as "%Ctrl") or fold increase versus the control group (as "fold induction") without 18S RNA
194 normalization . Data presented for plasma levels of glucose and insulin as well as for real-time PCR
195 of target gene expression are expressed as mean ± SEM (N = 8-10 for in vivo experiments & 4-8 for
196 in vitro experiments). Data for time-course studies were analyzed with two-way ANOVA followed by
197 Bonferroni post-hoc test with time and treatment as the two variables for statistical analysis. The data
198 for dose-dependence/drug treatment to elucidate post-receptor signaling were analyzed with one-way
199 ANOVA followed by Newman-Keuls test. Differences between groups were considered as significant
200 at $P < 0.05$.
201
202
203 **Results**
204
205 *Functional role of insulin in SPX regulation in vivo*
206
207 In goldfish entrained with one-meal-per-day feeding schedule, significant rises of glucose, insulin
208 and SPX levels were observed in the plasma with peaks at 1 hr after initiation of feeding (at time zero)
209 when compared to the time-matched unfed groups (Fig.1A-C). In the unfed groups, however, plasma
210 levels of glucose, insulin and SPX were reduced in a time-dependent manner with respect to their pre-
211 prandial basal. Pearson analysis of the data for plasma samples also revealed that positive correlation
212 could be noted between plasma levels of insulin and glucose (Fig.1D), SPX and glucose (Fig.1E) and
213 SPX and insulin (Fig. 1F), respectively. In goldfish, as revealed by RT-PCR, insulin was found to be
214 widely expressed in different tissues (e.g., the brain, gills, heart, gut, spleen, kidney, liver, muscle and
215 gonads) and brain areas (e.g., the olfactory bulb, telencephalon, optic tectum, hypothalamus, pituitary,
216 cerebellum, medulla oblongata and spinal cord), with the highest level of insulin signal detected in the
217 liver (Fig.2A). In the same feeding experiment, besides the rises in plasma insulin and SPX, rapid
218 elevation of insulin (Fig.2B) and SPX mRNA levels (Fig.2C) could also be observed in the liver with
219 peaks at 1 hr after food provision, suggesting that the liver may serve as a source of insulin and SPX

220 in circulation.

221

222 In parallel experiment, IP injection of goldfish with D-(+)-glucose could elevate plasma levels of
223 glucose (Fig.3A) with concurrent rises of insulin and SPX mRNA expression in the liver (Fig.3B &
224 3C). The treatment, however, did not induce notable changes in hepatic expression of IGF-I and IGF-
225 II transcripts (Supplemental Fig.1A). After IP injection of D-(+)-glucose, rapid rises of SPX mRNA
226 levels were also observed within the first 30 min in brain areas involved in feeding control, including
227 the hypothalamus (Fig.3D), telencephalon (Fig.3E) and optic tectum (Fig.3F). To examine the role of
228 insulin in SPX regulation, IP injection of human insulin was also performed in goldfish. Insulin of
229 human origin was used because (i) fish insulin with bioactivity is not commercially available, and (ii)
230 human insulin is highly homologous to goldfish counterpart (84% in B-domain & 78% in A-domain,
231 which form the mature peptide of insulin). In our study, IP injection with human insulin was shown to
232 reduce plasma levels of glucose in goldfish (Fig.4A), indicating that human insulin is fully functional
233 in fish model. Meanwhile, plasma levels of SPX (Fig.4B) as well as SPX mRNA expression in the
234 liver (Fig.4C) and brain areas including the hypothalamus (Fig.4D), telencephalon (Fig.4E) and optic
235 tectum (Fig.4F) were found to be elevated shortly after insulin treatment. To evaluate the functional
236 role of insulin in glucose-induced SPX release and gene expression, IP injection of D-(+)-glucose was
237 tested with co-treatment of the insulin antagonist S961. Similar to our time-course study, IP injection
238 of glucose consistently elevated plasma glucose (Fig.5A) and SPX levels (Fig.5B) with parallel rises
239 in SPX mRNA expression in the liver (Fig.5C) and brain areas including the hypothalamus (Fig.5D),
240 telencephalon (Fig.5E) and optic tectum (Fig.5F). These stimulatory effects, however, were found to
241 be suppressed/totally abolished by S961 co-treatment.

242

243 *SPX regulation by insulin in goldfish hepatocytes*

244

245 To shed light on SPX regulation by insulin at the hepatic level, primary culture of hepatocytes was
246 prepared from goldfish (Supplemental Fig.2A) and validation based on growth hormone-induced IGF-
247 I mRNA expression also confirmed that the cell culture was responsive to hormone stimulation under

serum-free condition (Supplemental Fig.2B), which is highly desirable for subsequent investigation of insulin action. As revealed by RT-PCR, expression of the receptors for insulin (including $InsR_1$ and $InsR_2$) and IGF-I (only for $IGF1R_2$ but not $IGF1R_1$) could be detected in the goldfish liver as well as hepatocyte culture (Fig.6A), implying that the hepatocytes can act as the regulatory target for the two endocrine factors. Under serum-free condition, static incubation of goldfish hepatocytes with D-(+)-glucose was found to elevate insulin (Fig.6B) and SPX mRNA levels (Fig.6C) in a dose-related manner without notable changes in IGF-I and -II gene expression (Supplemental Fig.1B). Parallel studies with insulin treatment also triggered a time- (Fig.6D) and dose-dependent elevation (Fig.6F) in SPX mRNA expression but similar treatment with glucagon was found to have no effects (Fig.6E).

In mammals, insulin and IGF-I are known to cross-react at receptor level (21). In our study, the receptor specificity for SPX regulation by insulin was also examined at the hepatic level. In goldfish hepatocytes, insulin induction was effective in triggering rapid phosphorylation of InsR (Fig.7A) and IGF1R (Fig.7B) with peaks at 15 min after drug treatment. At hepatocyte level, insulin-induced SPX mRNA expression was significantly suppressed by the InsR inhibitor HNMPA (by 83%) but similar treatment with the IGF1R blocker PPP could only induce a mild/marginal inhibition (by 16%) for the corresponding response (Fig.7C). However, the stimulatory effect of insulin could be totally abolished by PQ401, an inhibitor known to block InsR and IGR1R activation at the dose examined. In separate studies, co-treatment with the insulin antagonist S961 or InsR inhibitor HNMPA was also effective in blocking the stimulatory effect of D-(+)-glucose on SPX gene expression in goldfish hepatocytes (Fig. 7D), and again, a marginal inhibition could be noted with parallel inhibition using the IGF1R blocker PPP (data not shown). To shed light on the effect of InsR activation on SPX secretion at the hepatic level, a high-density culture system supplemented with Complete ™ protease inhibitor cocktail was established for goldfish hepatocytes. Based on our validation, the protease inhibitors did not alter the viability nor overall morphology of our cell culture (Supplemental Fig.3A) and with no notable effect on insulin, SPX and β-actin gene expression (Data not shown). Using this high-density cell culture, a 2-hr treatment with D-(+)-glucose (Supplemental Fig.3B) or insulin (Supplemental Fig.3C) was found to elevate SPX release and the stimulatory effect by D-(+)-glucose could be negated by co-treatment

with the InsR inhibitor HNMPA (Supplemental Fig.3D).

Given that InsR is known to be functionally coupled with MAPK and PI3/Akt pathways (22), the possible involvement of these post-receptor signaling cascades in SPX expression induced by insulin was also examined. Similar to the rapid phosphorylation of InsR, insulin treatment was effective in triggering protein phosphorylation of $MEK_{1/2}$, $ERK_{1/2}$, $MKK_{3/6}$, P_{38} ^{MAPK}, PI3K and Akt in goldfish hepatocytes with peaks at 15 min after drug administration (Fig.8A-C). In parallel studies with insulin treatment in the presence of inhibitors for various signaling targets, SPX mRNA expression caused by insulin stimulation was not affected by the $MEK_{1/2}$ inhibitor U1026 or PD98059 (Fig.9A) or $ERK_{1/2}$ blocker FR180204 or SCH772984 (Fig.9B). However, the SPX responses triggered by insulin could be reduced by simultaneous incubation with the P_{38} ^{MAPK} inhibitor PD169316 or SB203580 (Fig.9C), PI3K inactivator LY294002 or wortmannin (Fig.9D), Akt blocker API-2 or mTOR inhibitor rapamycin (Fig.9E). Furthermore, insulin-induced SPX gene expression could be totally abolished by exposing goldfish hepatocytes to the P_{38} ^{MAPK} inhibitor PD169316 and PI3K inactivator LY294002 at the same time (Fig.9F).

SPX regulation by insulin in goldfish brain cells

Since insulin treatment, similar to glucose challenge, could elevate SPX gene expression in vivo in the telencephalon, hypothalamus and optic tectum of the goldfish, goldfish brain cell culture was prepared from these brain areas (4) and used as a cell model to study the central actions of insulin on SPX regulation. In this cell culture, B27 supplement (with 4 mg/L insulin, Invitrogen) was used as a serum substitute to maintain a serum-free culture condition. To examine the effect of removing insulin intrinsic to the cell culture system, the serum substitute was replaced with an insulin-free preparation of B27 (Invitrogen). In this case, basal expression of SPX mRNA was reduced to a low level with no effect on β-actin gene expression (Fig.10A). In brain cells cultured with normal preparation of B27, insulin treatment was found to have the opposite effect and elevate SPX mRNA levels in a time- (Fig. 10B) and dose-related fashion (Fig.10C) with peak responses observed at 12 hr after initiation of drug

304 treatment.

305

306 As revealed by RT-PCR, transcript signals of the receptors for insulin (including $InsR_1$ and $InsR_2$)
307 and IGF-I (including $IGF1R_1$ and $IGF1R_2$) could be detected in the brain as well as brain cell culture
308 prepared from the goldfish (Fig.10D), implying that the central nervous system (CNS) is a regulatory
309 target for insulin and IGF-I in fish model. In goldfish brain cells, SPX mRNA expression induced by
310 insulin could be totally negated by the InsR inhibitor HNMPA while the parallel blockade with IGF1R
311 inactivator PPP could only yield a partial inhibition (~50%, Fig.10E). Regarding the post-receptor
312 signaling for insulin induction, similar to the results in hepatocytes, insulin was effective in triggering
313 rapid phosphorylation of $MEK_{1/2}$, $ERK_{1/2}$, $MKK_{3/6}$, P_{38} ^{MAPK}, PI3K and Akt in brain cell culture (Fig.
314 11A-C). Parallel experiments also revealed that SPX mRNA expression induced by insulin was not
315 sensitive to the blockade by the $MEK_{1/2}$ inhibitor PD98059 or $ERK_{1/2}$ blocker FR180204 (Fig.12A).
316 In contrast, the SPX responses could be obliterated by co-treatment of insulin with the P_{38} ^{MAPK}
317 inhibitor PD169316, PI3K inactivator LY294002, Akt blocker API-2 and mTOR inhibitor rapamycin,
318 respectively (Fig.12A & B).

319

320 As shown in our tissue profiling for insulin expression (Fig.2A), insulin signal was also located in
321 the brain of goldfish, especially in the hypothalamus, telencephalon and optic tectum. These findings
322 raise the possibility that insulin produced locally within the brain may play a role in SPX regulation
323 by glucose in the CNS. In our in vivo experiment, however, IP injection of D-(+)-glucose consistently
324 increased plasma insulin and insulin mRNA level in the liver (positive control of the study) but with
325 no effect on insulin gene expression in hypothalamus, telencephalon and optic tectum (Supplemental
326 Fig.4A). In parallel study with goldfish brain cell culture, similarly, treatment with D-(+)-glucose up
327 to 24 hr was found to be ineffective in altering basal levels of insulin and SPX mRNA expression
328 (Supplemental Fig.4B). These results suggest that insulin expression within the brain of goldfish is
329 not responsive to glucose changes in circulation. Probably, insulin from circulation rather than blood
330 glucose represents the major signal for SPX regulation within the CNS.

Discussion

SPX, a novel peptide identified by bioinformatics (1), has recently emerged as a regulatory factor with pleiotropic functions (see introduction for details). Since its association with energy balance and obesity has been reported in human and animal models (14) and plasma levels of SPX can be used as a marker for childhood obesity (15) and type II diabetes (6), the studies on SPX have aroused a lot of interest in the field. The research on SPX has become even more interesting with our recent study in goldfish revealing that central expression of SPX induced by food intake could act as a satiety signal to inhibit feeding behavior in fish model via modulation of orexigenic/anorexigenic factors expressed in brain areas involved in appetite control (4). Given that insulin, a key component of glucose homeostasis, is responsive to feeding, and by itself, also acts as an anorexigenic factor for signal integration of satiation and body adiposity (23), we postulate that it may be involved in SPX expression in fish model. In our feeding experiment, food intake could elevate plasma levels of glucose, insulin and SPX in goldfish with concurrent increase in insulin and SPX gene expression in the liver. Our results for the first time demonstrated that, besides the central expression of SPX, feeding could also induce a peripheral component of SPX, presumably through elevation of SPX production in the liver followed by SPX release into systemic circulation. Since (i) glucose-induced insulin release via ATP-sensitive K^+ channels (24) and sweet taste-sensing receptors (25) coupled to mitochondrial signal amplification (26) is well documented, and (ii) a positive correlation could be noted for plasma levels of glucose, insulin and SPX in our feeding experiment, it raises the possibility that glucose-induced insulin signals caused by feeding might play a role in SPX regulation. This idea is supported by our findings that (i) IP injection of glucose in goldfish could elevate plasma insulin and hepatic expression of insulin and SPX mRNA levels, and (ii) parallel treatment with insulin not only could induce SPX gene expression in the liver but also increase plasma levels of SPX. In both cases, SPX mRNA levels in brain areas involved in appetite control (i.e., hypothalamus, telencephalon & optic tectum) were also increased by glucose and insulin induction. Given that glucose-induced SPX secretion and SPX gene expression in the brain and liver were also sensitive to in vivo blockade by the insulin antagonist S961, it would be logical to conclude that the insulin signal caused by postprandial rise in blood glucose can contribute

to the SPX responses within the CNS and at the hepatic level in fish model.

In bony fish, endocrine pancreas exists as Brockman bodies scattering in the gut mesentery, e.g., in tilapia (27) and wolfish (28), while exocrine pancreas (mainly in the form of glandular acinus) can also spread into the liver leading to the formation of hepatopancreas in some species (29). Although hepatopancreas has been reported in the goldfish (30), the presence of an endocrine component of the pancreas has not been documented at the hepatic level. In our study, distinct structure for Brockman bodies could not be identified in the goldfish but the highest level of insulin expression was located in the liver. Furthermore, our experiments with IP injection of glucose in vivo and glucose treatment in hepatocytes in vitro have clearly shown that insulin signals not only could be detected at hepatic level but also inducible by glucose with parallel increase in SPX gene expression. In goldfish hepatocytes, glucose and insulin induction were both effective in elevating SPX mRNA levels and glucose-induced SPX secretion and gene expression could be negated by insulin antagonist S961 and/or InsR inhibitor HNMPA. These findings, taken together, suggest that (i) an insulin component responsive to glucose induction is present in the goldfish liver, and (ii) local production of insulin induced by glucose may act in an autocrine/paracrine manner to trigger SPX release/expression at hepatic level. In mammals, autocrine/paracrine actions of insulin have also been reported in the brain and involved in cognitive functions related to memory, learning and food anticipation (31). However, the local action of insulin produced at hepatic level has not been reported. Since glucagon did not alter SPX gene expression in our hepatocyte culture, it would be logical to assume that local production of glucose (e.g., by glycogenolysis/gluconeogenesis) may not have a major role in SPX regulation. Judging from the fact that the liver represents the largest organ in the body and serves as a major target for insulin, especially for glucose (32) and lipid homeostasis (33), the hepatopancreas in goldfish with an inducible component of insulin may also act as a source of insulin for both local actions as well as endocrine functions via blood circulation. Although Brockman's bodies could not be identified in goldfish, we do not exclude the possibility that postprandial rise in blood glucose could also induce insulin release from goldfish pancreas which may also contribute to SPX regulation in the liver as well as in brain areas involved in appetite control.

Of note, glucose treatment, both in vivo and in vitro, did not alter hepatic expression of IGF-I/-II mRNA levels in our studies, the possible involvement of local production of IGFs for SPX regulation is rather unlikely. In mammals, insulin is known to cross-react with IGF1R (21) and InsR and IGF1R are functionally coupled with MAPK and PI3K/Akt pathways (34). In some tissues (e.g., placenta), InsR and IGF1R can also form hybrid receptors (35) with higher affinity for IGF-I (36). In bony fish, isoforms of InsR (37) and IGF1R (38) have been cloned and believed to be the result of whole genome duplication occurred during the evolution of teleost lineage (39). In goldfish hepatocytes, two forms of InsR ($InsR_1$ & $InsR_2$) and one form of IGF1R ($IGF1R_2$) could be identified by RT-PCR and insulin exposure was found to induce rapid phosphorylation of InsR and IGF1R, suggesting that the receptors for insulin and IGF-I may play a role in mediating insulin functions at the hepatic level. Given that (i) the InsR inhibitor HNMPA was notably more effective (83% blockade) than the IGF1R inhibitor PPP (16% blockade) in attenuating insulin-induced SPX mRNA expression in goldfish hepatocytes, and (ii) the SPX responses caused by insulin was totally abolished by PQ401, an inhibitor known to inactivate both InsR and IGF1R at the dose tested, it is conceivable that the two receptors are both involved in SPX regulation at hepatic level with InsR as the dominant form mediating insulin action. Regarding the post-receptor signaling mechanisms for SPX regulation, insulin was capable of triggering $MEK_{1/2}$, $ERK_{1/2}$, $MKK_{3/6}$, P_{38} ^{MAPK}, PI3K and Akt phosphorylation in goldfish hepatocytes. These results are comparable with the recent report on InsR signaling in trout adipocytes (40) and consistent with the idea that InsR signaling via MAPK and PI3K/Akt cascades is well conserved in vertebrate evolution (41). In goldfish hepatocytes, however, insulin-induced SPX mRNA expression was not affected by the inhibitors for $MEK_{1/2}$ (U0126 & PD98059) or $ERK_{1/2}$ (FR18024 & SCH772984), but attenuated by the inhibitors for P_{38} ^{MAPK} (PD169316 & SB20380), PI3K (LY294002 & Wormannin), Akt (API-2) and mTOR (Rapamycin), respectively. Besides, the SPX responses caused by insulin could be totally negated by co-treatment with the inhibitors for P_{38} ^{MAPK} (PD169316) and PI3K (LY294002), suggesting that the $MKK_{3/6}/P_{38}$ ^{MAPK} and PI3K/Akt/mTOR but not $MEK_{1/2}/ERK_{1/2}$ cascades are involved in SPX gene expression induced by insulin at the hepatic level in fish model.

As mentioned in the preceding section, insulin signals induced by glucose could also trigger SPX gene expression in brain areas involved in appetite control, which might initiate the satiation response after feeding in goldfish via differential regulation of orexigenic/anorexigenic signals within the CNS. This idea is comparable to the model in mammals with central regulation of energy balance and body weight via modulation of feeding regulators (e.g., NPY & POMC) within the hypothalamus by satiety signals (e.g., leptin) (42). In our study, the effect of insulin on central expression of SPX was further substantiated by in vitro experiments with goldfish brain cells, in which (i) removing insulin from the cell culture system (using insulin-free B27) could suppress basal level of SPX mRNA, and (ii) insulin treatment, in contrast, could up-regulate SPX gene expression in a time- and dose-related manner. Our findings are also in agreement with the previous reports (e.g., in rodents) on (i) insulin uptake into the CNS by transcytosis across choroid plexus via InsR coupled to LRP2/megalin (43) and (ii) the role of insulin as an anorexigenic factor via its central actions in modulating NPY, AgRP, POMC and MCR4 expression (44). Apparently, SPX can serve as a novel component for the central actions of insulin on appetite control. Since insulin expression within the brain in goldfish was not responsive to glucose in vivo and in vitro, it is likely that insulin from circulation rather than blood glucose represents the key signal triggering SPX expression within the CNS in fish model. Regarding the receptor specificity for insulin's action within the CNS, the results of RT-PCR revealed that multiple isoforms of InsR ($InsR_1$ & $InsR_2$) and IGF1R ($IGF1R_1$ & $IGF1R_2$) could be detected in the whole brain as well as brain cell culture prepared from the goldfish. Similar to goldfish hepatocytes, insulin-induced SPX mRNA expression in brain cells was found to be more sensitive to the blockade by the InsR inhibitor HNMPA (~100%) compared to similar treatment with the IGF1R inhibitor PPP (~50% blockade). These results again suggest that the central effect of insulin on SPX expression is mediated mainly by InsR and to a lower extent by IGF1R. Unlike the brain, $IGF1R_1$ was undetectable in goldfish liver/hepatocytes and $IGF1R_2$ was the only IGF1R identified at the hepatic level, implying that different isoforms of IGF1R are expressed in a tissue-specific manner. Whether it can also contribute to the tissue-specific actions of IGFs as recently reported for insulin/IGF-I signaling in body metabolism and aging in rodents (45) is unclear and remains to be investigated. Although insulin was effective in triggering protein phosphorylation of $MEK_{1/2}$, $ERK_{1/2}$, $MKK_{3/6}$, P_{38} ^{MAPK}, PI3K and Akt in goldfish brain cells, SPX mRNA

443 expression induced by insulin in the cell culture was not sensitive to the blockade of $MEK_{1/2}$ (e.g., by
444 PD98059) and $ERK_{1/2}$ (e.g., by FR180204) but could be totally abolished by inhibiting P_{38} ^{MAPK} (e.g.,
445 by PD169316), PI3K (e.g., by LY294002), Akt (e.g., by API-2) and mTOR (e.g., by Rapamycin),
446 respectively. These findings as a whole confirm that, similar to the case of goldfish hepatocytes, the
447 central effect of insulin on SPX regulation is also mediated by $MKK_{3/6}/P_{38}$ ^{MAPK} and PI3K/Akt/mTOR
448 but not $MEK_{1/2}/ERK_{1/2}$ cascades.
449
450 In summary, using goldfish as animal model, we have demonstrated for the first time that insulin
451 signals induced by postprandial rise in blood glucose could serve as the functional link between food
452 intake and SPX expression both in the central (i.e., within CNS) and periphery (i.e., in the liver). In
453 our study, we also have the novel finding that the liver in goldfish represents a major site of insulin
454 expression, which is also inducible by changes in glucose levels. In our working model (Fig.13), food
455 intake in goldfish can induce insulin expression in the liver via elevation in blood glucose (probably
456 with parallel rise in insulin signal from the pancreatic). Local production of insulin acts in an auto-
457 crine/paracrine manner to increase SPX expression at the hepatic level via InsR (to a lesser extent via
458 IGF1R) coupled to the $MKK_{3/6}/P_{38}$ ^{MAPK} and PI3K/Akt pathways. Hepatic output of SPX also elevates
459 SPX level in circulation and constitutes a peripheral SPX signal acting on the CNS. Meanwhile,
460 insulin released from the liver (together with pancreatic insulin) can elevate insulin level in blood and
461 serve as an endocrine signal to induce SPX expression in brain areas involved in feeding behavior.
462 Apparently, the central effect of insulin on SPX expression is also mediated through InsR coupled with
463 $MKK_{3/6}/P_{38}$ ^{MAPK} and PI3K/Akt signaling. Since our previous study with ICV injection of SPX in
464 goldfish has confirmed that (i) SPX by acting centrally could inhibit feeding behavior and food intake,
465 and (ii) these inhibitory effects were mediated by SPX inhibition of NPY and AgRP with concurrent
466 stimulation of CART, CCK and POMC expression in brain areas involved in appetite control (4), local
467 production of SPX within the CNS together with the SPX input from the periphery induced by insulin
468 presumably can inhibit feeding behavior in goldfish via differential actions on orexigenic/anorexigenic
469 signals within the feeding circuitry in the brain. Our study, as a whole, has provided new insights on
470 the mechanisms and signal transduction for the role of SPX as a satiety factor in fish model. Of note,

plasma SPX is closely associated with glucose homeostasis (e.g., in diabetic patient) (6) and fatty acid uptake/body adiposity (e.g., in obese mouse) (14). The role of circulating SPX induced by feeding in energy balance and glucose/lipid metabolism for sure will be an interesting topic for further perusal. Recently, type II and III galanin receptors have been proposed to be the cognate receptor for SPX, and interestingly, type III galanin receptor could not be found in the genome of fish species, e.g., zebrafish (17), research is now underway in our laboratory to study the receptor specificity as well as the post-receptor signaling mechanisms for SPX-induced satiation and regulation of orexigenic/anorexigenic factors expressed within the brain in goldfish.

(5768 words)

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613 **Legends**

614

615 Fig.1 Effects of food intake on plasma levels of glucose, insulin and SPX in goldfish. Goldfish
616 entrained with a one-meal-per-day feeding schedule (with 8 fish/group) was provided with fish feed at
617 10:00 AM (taken as 0 hr as marked by the arrow) in treatment group (as "fed" group) but without food
618 provision in the control group (as "unfed" group). Blood samples were harvested from caudal
619 vasculature in individual fish at the time points as indicated and subjected to respective assays for the
620 measurement of plasma glucose (A), insulin (B) and SPX (C). Pearson analysis was also performed
621 with the plasma data for glucose and insulin (D), glucose and SPX (E), and insulin and SPX (F),
622 respectively. Except for Pearson analysis (with raw data), data presented are expressed as mean ±
623 SEM (N = 8) and groups denoted by different letters represent a significant difference at $P < 0.05$.

624

625 Fig.2 Tissue expression profile of insulin in goldfish and postprandial changes in hepatic expression
626 of insulin and SPX. (A) RT-PCR of insulin expression in selected tissues and brain areas in goldfish.
627 Total RNA was isolated from the tissue/brain area as indicated and subjected to RT-PCR with primers
628 specific for goldfish insulin. The authenticity of PCR product was confirmed by PCR Southern using
629 a DIG-labeled probe for goldfish insulin and RT-PCR for β-actin was used as internal control. Effects
630 of food intake on (B) insulin and (C) SPX gene expression in the liver of goldfish. In the same study
631 described in Fig.1, total RNA was isolated from the liver at the respective time points in the "fed" and
632 "unfed" groups and subjected to real-time PCR for insulin and SPX mRNA measurement. Parallel
633 measurement of 18S RNA was performed to serve as the internal control for data normalization. Data
634 presented for the temporal changes of insulin and SPX mRNA levels are expressed as mean ± SEM
635 (N = 8) and groups denoted by different letters represent a significant difference at $P < 0.05$.

636

637 Fig.3 In vivo treatment with glucose on central and peripheral expression of SPX in goldfish. Fish
638 entrained with one-meal-per-day feeding schedule (8 fish/group) were IP injected with D-(+)-glucose
639 (1.5 mg/g BW) at 10:00 AM (taken as 0 hr) without food provision. Parallel injection with physio-
640 logical saline was used as the control. At the time points as indicated, blood samples were collected

for measurement of plasma glucose (A). For peripheral tissue, total RNA was isolated from the liver at the respective time points for real-time PCR of (B) insulin and (C) SPX mRNA expressed at the hepatic level. For central expression of SPX, RNA samples were isolated from (D) the hypothalamus, (E) telencephalon and (F) optic tectum respectively and used for SPX mRNA measurement in selected brain areas. In this study, parallel measurement of 18S RNA was used as the internal control. Data presented are expressed as mean ± SEM (N = 8) and groups denoted by different letters represent a significant difference at $P < 0.05$.

Fig.4 In vivo treatment with insulin on central and peripheral expression of SPX in goldfish. Fish entrained with one-meal-per-day feeding schedule (8 fish/group) were IP injected with insulin (4 USP units/g BW) at 10:00 AM (taken as 0 hr) without food provision. Parallel injection with physiological saline was used as the control. At the time points as indicated, blood samples were collected for plasma glucose (A) and SPX measurement (B). Meanwhile, total RNA was isolated from the liver (C) and brain areas including the hypothalamus (D), telencephalon (E) and optic tectum (F) for real-time PCR of SPX mRNA measurement. Parallel measurement of 18S RNA was also conducted to serve as the internal control. Data presented are expressed as mean ± SEM (N = 8) and groups denoted by different letters represent a significant difference at $P < 0.05$.

Fig.5 In vivo blockade by insulin antagonist on glucose-induced central and peripheral expression of SPX in goldfish. Goldfish entrained with one-meal-per-day feeding schedule (10 fish/group) were IP injected with D-(+)-glucose (1.5 mg/g BW) at 10:00 AM (without food provision) with or without co-injection of the insulin antagonist S961 (30 nmole/kg BW). Parallel injection with saline was used as the control. Blood sampling and tissue collection were conducted 30 min after drug treatment. Plasma samples prepared were used for measurement of plasma glucose (A) and SPX levels (B). For peripheral expression of SPX, total RNA was isolated from the liver for real-time PCR of SPX mRNA expression at the hepatic level (C). For central expression of SPX, RNA samples were isolated from the hypothalamus (D), telencephalon (E) and optic tectum (F), respectively, and used for SPX mRNA measurement in selected brain areas. In this study, parallel measurement of 18S RNA was used as the

internal control. Data presented are expressed as mean ± SEM (N = 10) and groups denoted by different letters represent a significant difference at $P < 0.05$.

Fig.6 Functional role of glucose and insulin on SPX gene expression in goldfish hepatocytes. InsR and IGF1R expression at the hepatic level: (A) RT-PCR of different isoforms of InsR ($InsR_1$ & $InsR_2$) and IGF1R ($IGF1R_1$ & $IGF1R_2$) expressed in the liver and hepatocyte culture prepared from goldfish using primers for the respective gene targets. RT-PCR for InsR and IGF1R expressed in muscle was used as positive control and parallel PCR for β-actin was used as internal control. Regulation of SPX mRNA expression in primary culture of goldfish hepatocytes: Dose-dependence of glucose treatment on (B) insulin and (C) SPX mRNA expression in goldfish hepatocytes. Hepatocytes were treated for 48 hr with increasing concentrations of D-(+)-glucose and subjected to RNA isolation and real-time PCR for insulin and SPX mRNA measurement. Time course of (D) insulin and (E) glucagon treatment on SPX mRNA expression. Goldfish hepatocytes were incubated with insulin (100 nM) or glucagon (100 nM) for the duration as indicated. Dose-dependence of insulin (F) on SPX mRNA expression was also examined in the cell culture with duration of drug treatment fixed at 48 hr. Data presented are expressed as mean ± SEM (N = 8) and groups denoted by different letters represent a significant difference at $P < 0.05$.

Fig.7 Receptor specificity of SPX regulation by insulin in goldfish hepatocytes. Effects of insulin on (A) InsR and (B) IGF1R phosphorylation in goldfish hepatocytes. Cell culture was incubated with insulin (10 nM) for the duration as indicated. After that, cell lysate prepared was used for Western blot with antibodies specific for phosphorylated form ("P-") and total protein ("T-") of InsR and IGF1R, respectively. In this experiment, Western blot for β-actin was used as the loading control. The results of Western blot were shown in the panels on the left while the quantitative data expressed as the ratio of the signal intensity (registered by densitometric scanning using ImageJ) of the P-form over the T-form of target protein were presented in the right panels. (C) Effects of blocking InsR and IGF1R on insulin-induced SPX mRNA expression. Goldfish hepatocytes were exposed to insulin (10 nM) for 48 hr with/without the IGF1R inhibitor PPP (10 μM), InsR inhibitor HNMPA (10 μM) or non-selective

InsR/IGF1R inhibitor PQ401 (50 μM). (D) Insulin blockade on glucose-induced SPX gene expression at the hepatic level. Hepatocytes were exposed to D-(+)-glucose (6 g/L) for 48 hr with co-treatment of the insulin antagonist S961 (500 nM) or InsR inhibitor HNMPA (10 μM). After drug treatment, total RNA was isolated and subjected to real-time PCR for SPX mRNA measurement. Data presented are expressed as mean ± SEM (N = 4 for Western blot and N = 8 for experiments with inhibitors) and groups denoted by different letters represent a significant difference at $P < 0.05$.

Fig.8 Effects of insulin on MAPK and PI3K/Akt signaling in goldfish hepatocytes. Hepatocytes were treated with insulin (10 nM) for the duration as indicated. After that, cell lysate was prepared and subjected to Western blot using antibodies for phosphorylated form ("P-") and total protein ("T-") of $MEK_{1/2}$ and $ERK_{1/2}$ (A), $MKK_{3/6}$ and P_{38} ^{MAPK} (B), and PI3K and Akt (C), respectively. Parallel Western blot for β-actin was used as the loading control. Representative results of Western blot were shown in the panels on the left while the quantitative data expressed as the ratio of the signal intensity (registered by densitometric scanning using ImageJ) of the P-form over the T-form of target protein were presented in the right panels. Data presented are expressed as mean ± SEM (N = 4) and groups denoted by different letters represent a significant difference at $P < 0.05$.

Fig.9 Blocking MAPK and PI3K/Akt signaling cascades on insulin-induced SPX mRNA expression in goldfish hepatocytes. Hepatocytes were incubated with insulin (10 nM) for 48 hr in the presence of (A) the $MEK_{1/2}$ inhibitor U0126 (10 μM) or PD98059 (10 μM), (B) the $ERK_{1/2}$ inhibitor FR180204 (2 μM) or SCH772984 (10 nM), (C) the P_{38} ^{MAPK} inhibitor PD169316 (10 μM) or SB203580 (10 μM), (D) the PI3K inhibitor LY294002 (10 μM) or Wortmannin (100 nM), and (E) the Akt inhibitor API-2 (20 μM) or mTOR inhibitor Rapamycin (20 nM)), respectively. In parallel study, insulin induction (10 nM) was also tested with co-treatment of $MEK_{1/2}$ inhibitor PD169316 (10 μM) and PI3K inhibitor LY294002 (10 μM) simultaneously (F). After drug treatment, total RNA was isolated and subjected to real-time PCR for SPX mRNA measurement. Data presented are expressed as mean ± SEM (N = 8) and groups denoted by different letters represent a significant difference at $P < 0.05$.

Fig.10 Functional role of insulin on SPX gene expression in goldfish brain cells. (A) Removal of insulin from culture medium on SPX mRNA expression in primary culture of goldfish brain cells. In this study, brain cells were cultured in medium supplemented with B27 (a serum substitute with 4 mg/L insulin) or insulin-free B27 (for removing insulin from cell culture) for the duration as indicated. In parallel experiments, time course (B) and dose-dependence (C) of insulin treatment on SPX mRNA expression in goldfish brain cells were also examined in culture medium containing regular B27 (with insulin). For time course study, static incubation with insulin (30 nM) was conducted for the duration as indicated. For dose-dependence, brain cells were treated for 12 hr with increasing levels of insulin. To evaluate the receptor specificity for SPX mRNA expression induced by insulin within the CNS of goldfish, RT-PCR of different isoforms of InsR ($InsR_1$ & $InsR_2$) and IGF1R ($IGF1R_1$ & $IGF1R_2$) was conducted in the brain as well as brain cells prepared from goldfish (D) using primers specific for the respective gene targets. RT-PCR for InsR and IGF1R expressed in muscle was used as the positive control while parallel PCR for β-actin was used as internal control. For functional studies, blockade of InsR and IGF1R on insulin-induced SPX mRNA expression were also tested in goldfish brain cells (E). In this case, brain cells were incubated with insulin (30 nM) for 12 hr in the presence/absence of the InsR inhibitor HNMPA (10 μM) or IGF1R inhibitor PPP (10 μM). After treatment, total RNA was isolated and subjected to real-time PCR for SPX mRNA measurement. Parallel measurement of β-actin mRNA was used as the internal control. Data presented are expressed as mean ± SEM (N = 8) and groups denoted by different letters represent a significant difference at $P < 0.05$.

Fig.11 Effects of insulin on MAPK and PI3K/Akt signaling in goldfish brain cells. Brain cells were challenged with insulin (10 nM) for the duration as indicated. After that, cell lysate was prepared and subjected to Western blot using antibodies for phosphorylated form ("P-") and total protein ("T-") of $MEK_{1/2}$ and $ERK_{1/2}$ (A), $MKK_{3/6}$ and P_{38} ^{MAPK} (B), and PI3K and Akt (C), respectively. Parallel Western blot for β-actin was used as the loading control. Representative results of Western blot were shown in the panels on the left while the quantitative data expressed as the ratio of the signal intensity (registered by densitometric scanning using ImageJ) of the P-form over the T-form of target protein were presented in the right panels. Data presented are expressed as mean ± SEM (N = 4) and groups

753 denoted by different letters represent a significant difference at $P < 0.05$.

754

755 Fig.12 Blocking MAPK and PI3K/Akt signaling cascade on insulin-induced SPX mRNA expression
756 in goldfish brain cells. Primary culture of goldfish brain cells was incubated with insulin (30 nM) for
757 12 hr with/without (A) the $MEK_{1/2}$ inhibitor PD98059 (10 μM), $ERK_{1/2}$ inhibitor FR180204 (2 μM) or
758 P_{38} ^{MAPK} inhibitor PD169316 (10 μM)), or (B) PI3K inhibitor LY294002 (10 μM), Akt inhibitor API-2
759 (20 μM) or mTOR inhibitor Rapamycin (20 nM)), respectively. After drug treatment, total RNA was
760 isolated and subjected to real-time PCR for SPX mRNA measurement. In these experiments, parallel
761 measurement of β-actin mRNA was used as internal control. Data presented are expressed as mean ±
762 SEM (N = 8) and groups denoted by different letters represent a significant difference at $P < 0.05$.

763

764 Fig.13 Working model for insulin acting as a functional link between feeding and SPX expression in
765 the liver as well as in the brain of fish model. In goldfish, food intake can induce insulin expression in
766 the liver via elevation in blood glucose (probably with parallel rise in insulin signal from the pancreas).
767 Local production of insulin acts in an autocrine/paracrine manner to up-regulate SPX expression at
768 the hepatic level mainly via InsR (to a lesser extent by IGF1R) functionally coupled with the $MKK_{3/6}$/
769 P_{38} ^{MAPK} and PI3K/Akt pathways. SPX produced by the liver enters the systemic circulation and serves
770 as a peripheral signal acting on the CNS. Meanwhile, insulin released from the liver (together with
771 insulin from the pancreas) can elevate insulin level in circulation and exerts a central effect to induce
772 SPX expression in brain areas involved in feeding control via InsR coupled to $MKK_{3/6}$/P_{38} ^{MAPK} and
773 PI3K/Akt signaling. The combined effect of central expression of SPX together with the peripheral
774 input of SPX can differentially regulate orexigenic and anorexigenic signals expressed in the feeding
775 circuitry within the CNS and lead to an inhibition on feeding behavior and subsequent suppression of
776 food intake. These biological changes are believed to play a role in the initiation of satiation response
777 after feeding in fish model.

778

Supplemental Table (1)

Primer sequences, PCR conditions and QC parameters for real-time PCR of target genes.

Gene name / Genbank accession no **Sequences of forward & reverse primers**	**Real-time PCR condition**					**Product size & *Tm* value**
	Denaturing	Annealing	Extension	Detection	Cycle no.	
Spexin / JQ894857.1 Forward: 5’ TAATGTTTCAAGTTCATCCTC 3’ Reverse: 5’ AGTGGTTATTTCACAGATCAGTCC 3’	94 ℃ 30 sec	55 ℃ 30 sec	72 ℃ 30 sec	80 ℃ 20 sec	×35	156 bp (*Tm* = 86 ℃)
Insulin / KT071542.1 Forward: 5’ GATGCCCTCTACCTGGTCTG 3’ Reverse: 5’ TCCTTATCAGCTCTGCGTGA 3’	94 ℃ 30 sec	64 ℃ 30 sec	72 ℃ 30 sec	84 ℃ 20 sec	×35	157 bp (*Tm* = 88 ℃)
IGF-I / GU583648.1 Forward: 5’ TTCAAGTGTACCATGCGCTG 3’ Reverse: 5’ ACCGTCTTGAATTAGGCCCA 3’	94 ℃ 30 sec	62 ℃ 30 sec	72 ℃ 30 sec	88 ℃ 20 sec	×35	201 bp (*Tm* = 93 ℃)
IGF-II / FJ410929.1 Forward: 5’ AGACCCTTTGCGGTGGAGA 3’ Reverse: 5’ GGAAACATCTCGCTCGGACT 3’	94 ℃ 30 sec	62 ℃ 30 sec	72 ℃ 30 sec	84 ℃ 20 sec	×35	200 bp (*Tm* = 90 ℃)
18S RNA / HQ615531.1 Forward: 5’AGCAACTTTAGTATACGCTATTGGAG 3’ Reverse: 5’ CCTGAGAAACGGCTACCACATCC 3’	94 ℃ 30 sec	56 ℃ 30 sec	72 ℃ 30 sec	87 ℃ 20 sec	×30	285 bp (*Tm* = 91 ℃)

Supplemental Table (2)

Primer sequences, PCR conditions and QC parameters for RT-PCR of target genes.

Gene name / Genbank accession no **Sequences of forward & reverse primers**	**PCR condition**				**Product size**
	Denaturing	Annealing	Extension	Cycle no.	
Insulin / KT071542.1 Forward: 5’ GATGCCCTCTACCTGGTCTG 3’ Reverse: 5’ TCCTTATCAGCTCTGCGTGA 3’	94 ℃ 30 sec	64 ℃ 30 sec	72 ℃ 40 sec	×35	157 bp
$InsR_1$ / AF218355.1 Forward: 5’ AGGAAGAAACAAACCGAAGGACC 3’ Reverse: 5’ ACACCGAGCAAGCGAAGCAC 3’	94 ℃ 30 sec	62 ℃ 30 sec	72 ℃ 40 sec	×35	323 bp
$InsR_2$ / AF321225.1 Forward: 5’ GAAGAAAGAGGCGGAGGAGAC 3’ Reverse: 5’ CAGATTGGAGATGACCGTTGACT 3’	94 ℃ 30 sec	59 ℃ 30 sec	72 ℃ 40 sec	×35	252 bp
$IGF1R_1$ / AF216772.2 Forward: 5’ ACCAGAGCGGCAGATAGAGC 3’ Reverse: 5’ GGATGATGATGTAGGACAGGTAGTG 3’	94 ℃ 30 sec	59 ℃ 30 sec	72 ℃ 40 sec	×35	352 bp
$IGF1R_2$ / AF216773.2 Forward: 5’ CATCGCAAAGGGCGTCGTCA 3’ Reverse: 5’ TGCCATCCCGTCCGCAATCT 3’	94 ℃ 30 sec	67 ℃ 30 sec	72 ℃ 40 sec	×35	325 bp
β-actin / AB039726.2 Forward: 5’ CTGGTATTCGTGATGGACTCT 3’ Reverse: 5’ AGCTCATAGCTCTTCTCCAG 3’	94 ℃ 30 sec	56 ℃ 30 sec	72 ℃ 40 sec	×30	285 bp

Fig.1

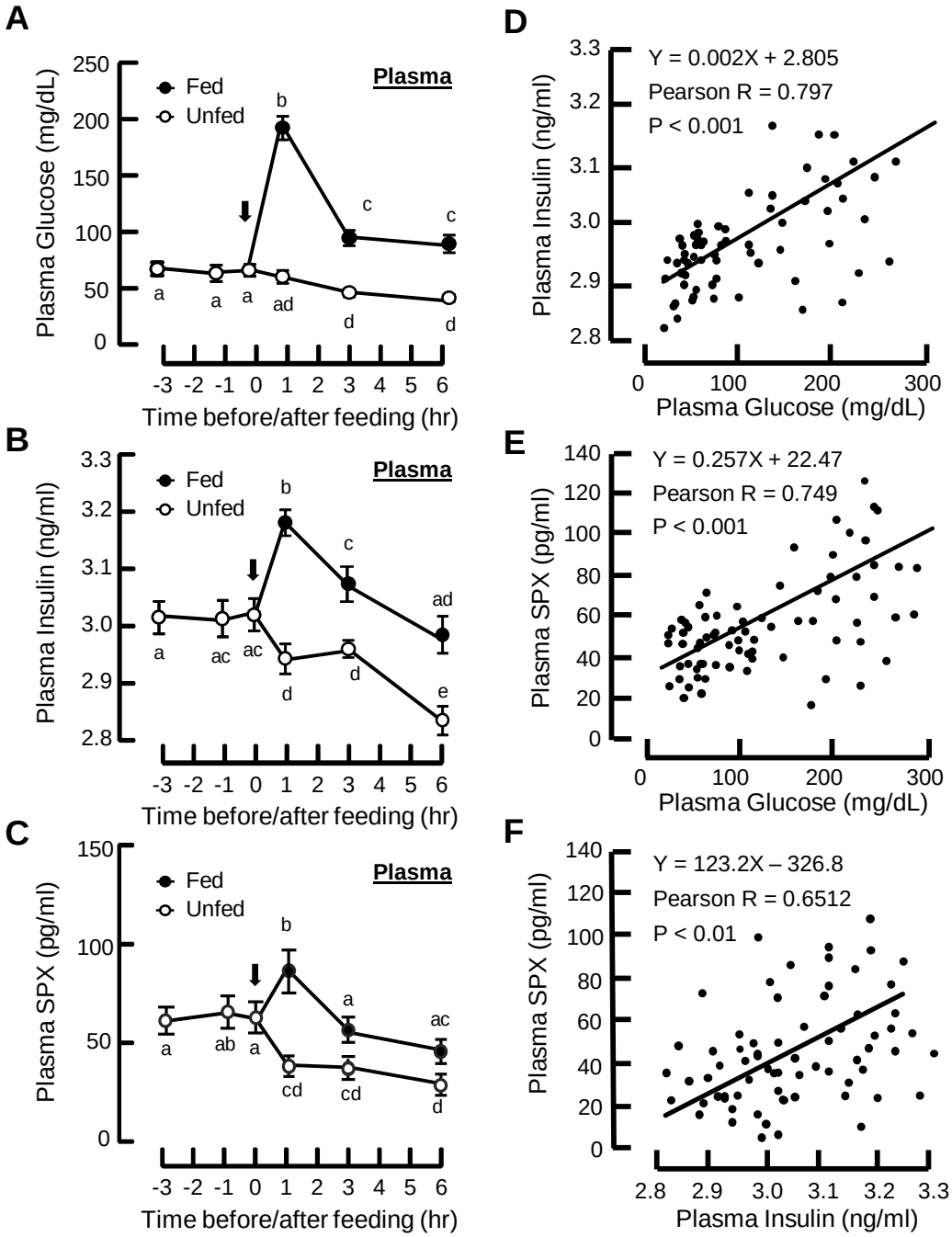


Fig.2

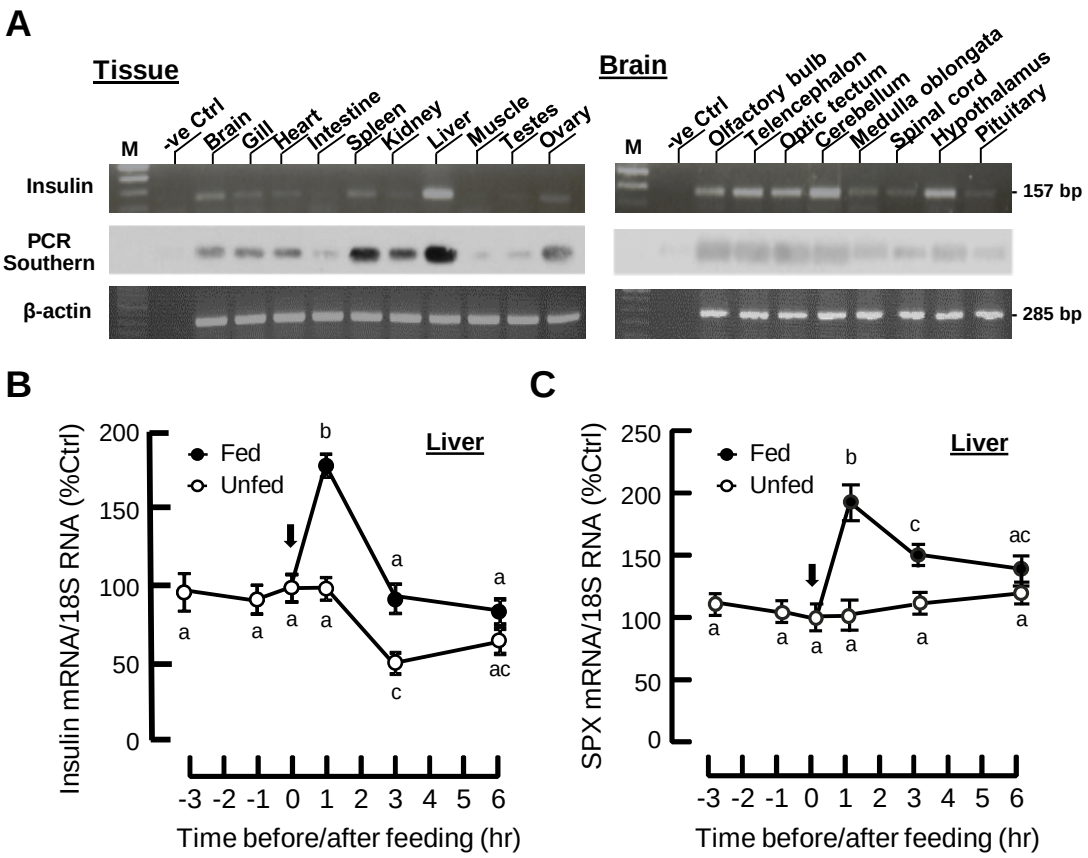
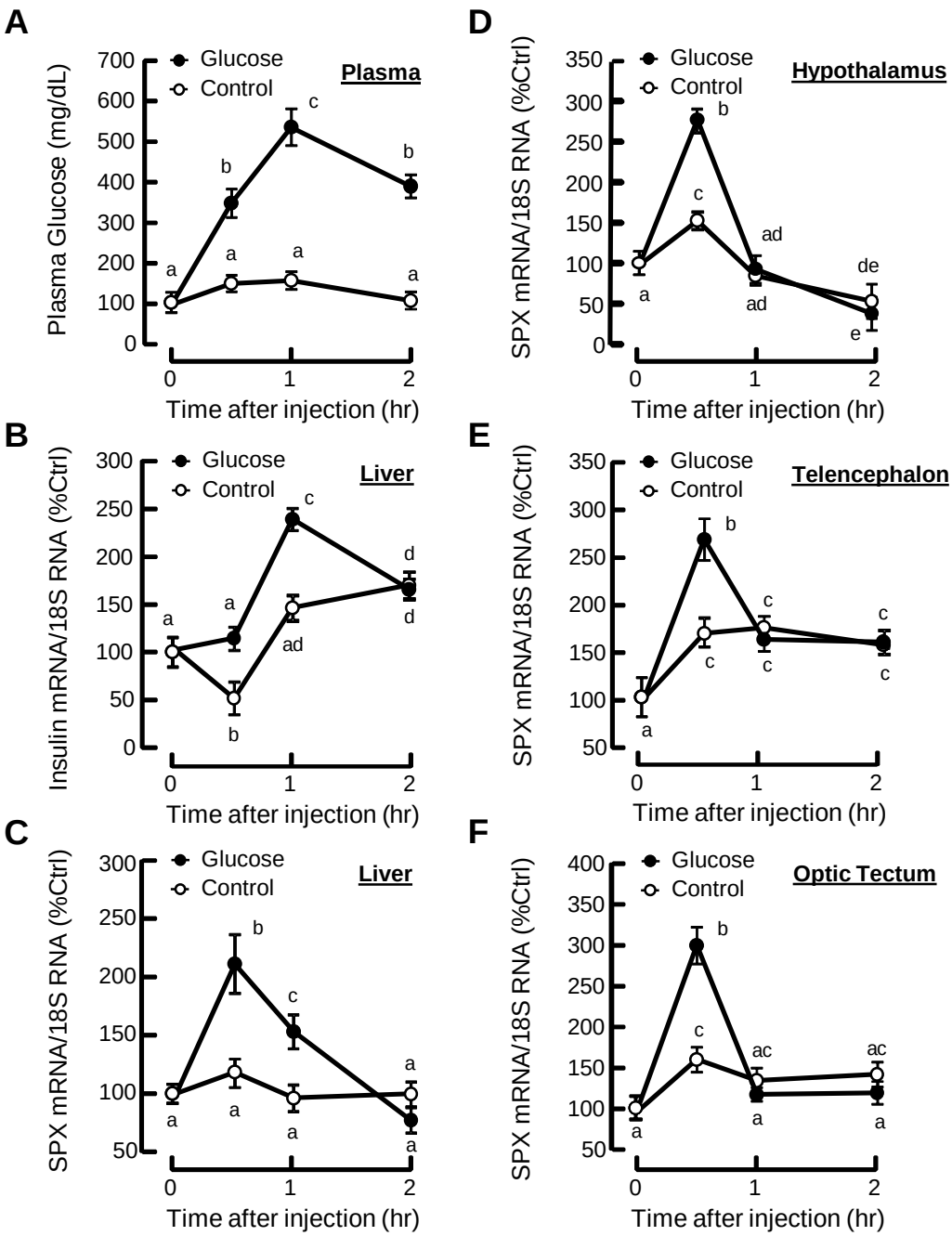


Fig.3



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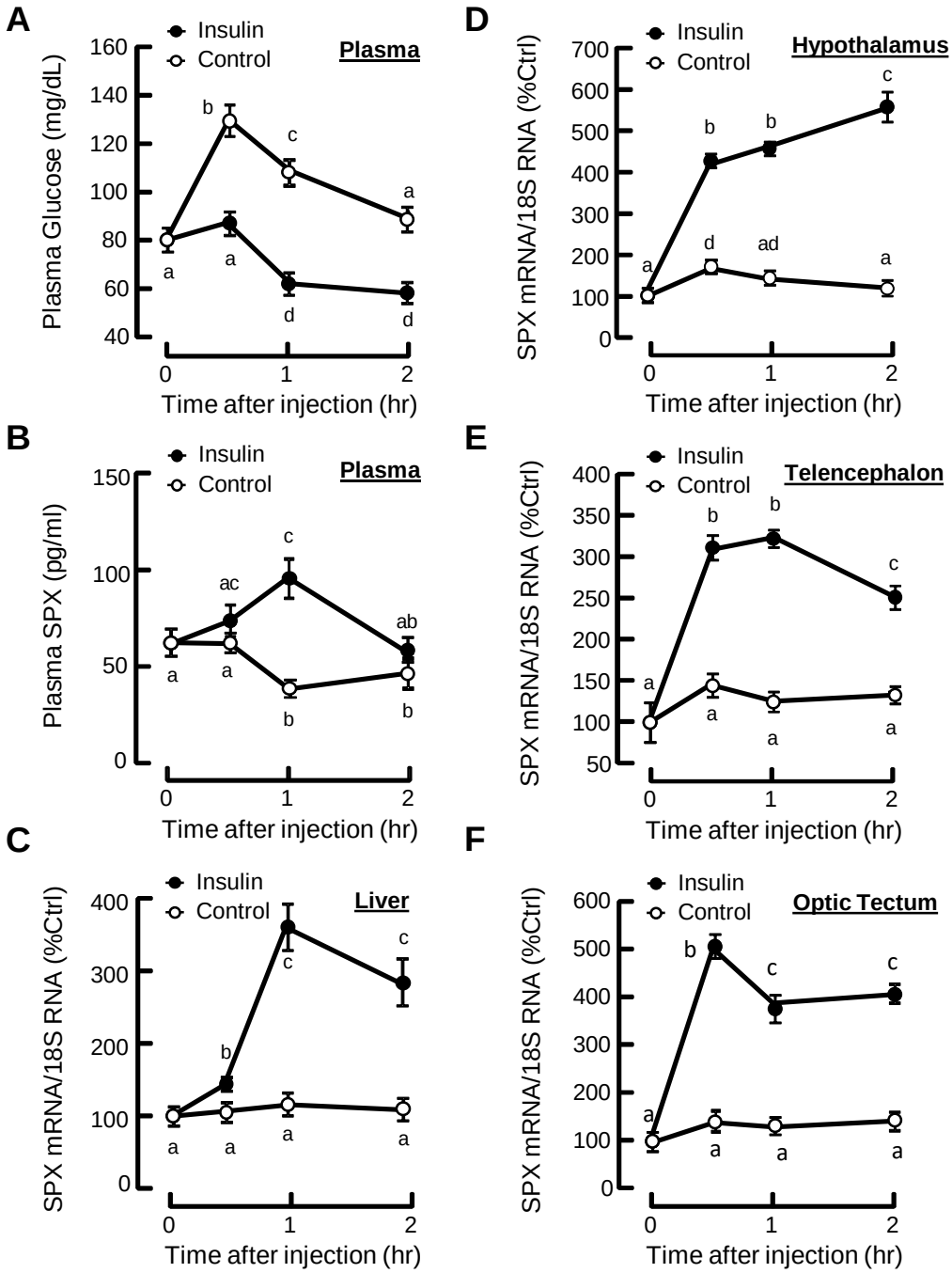

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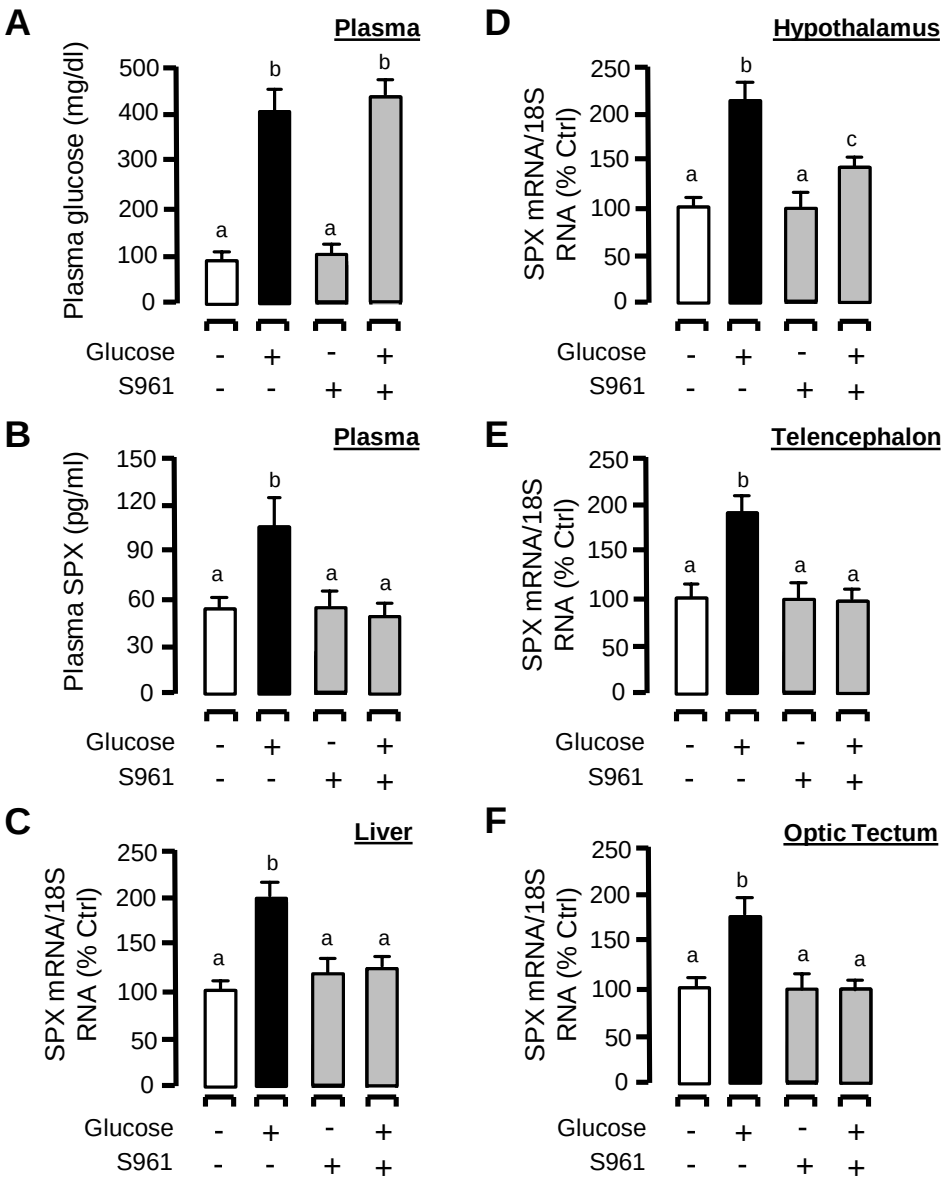

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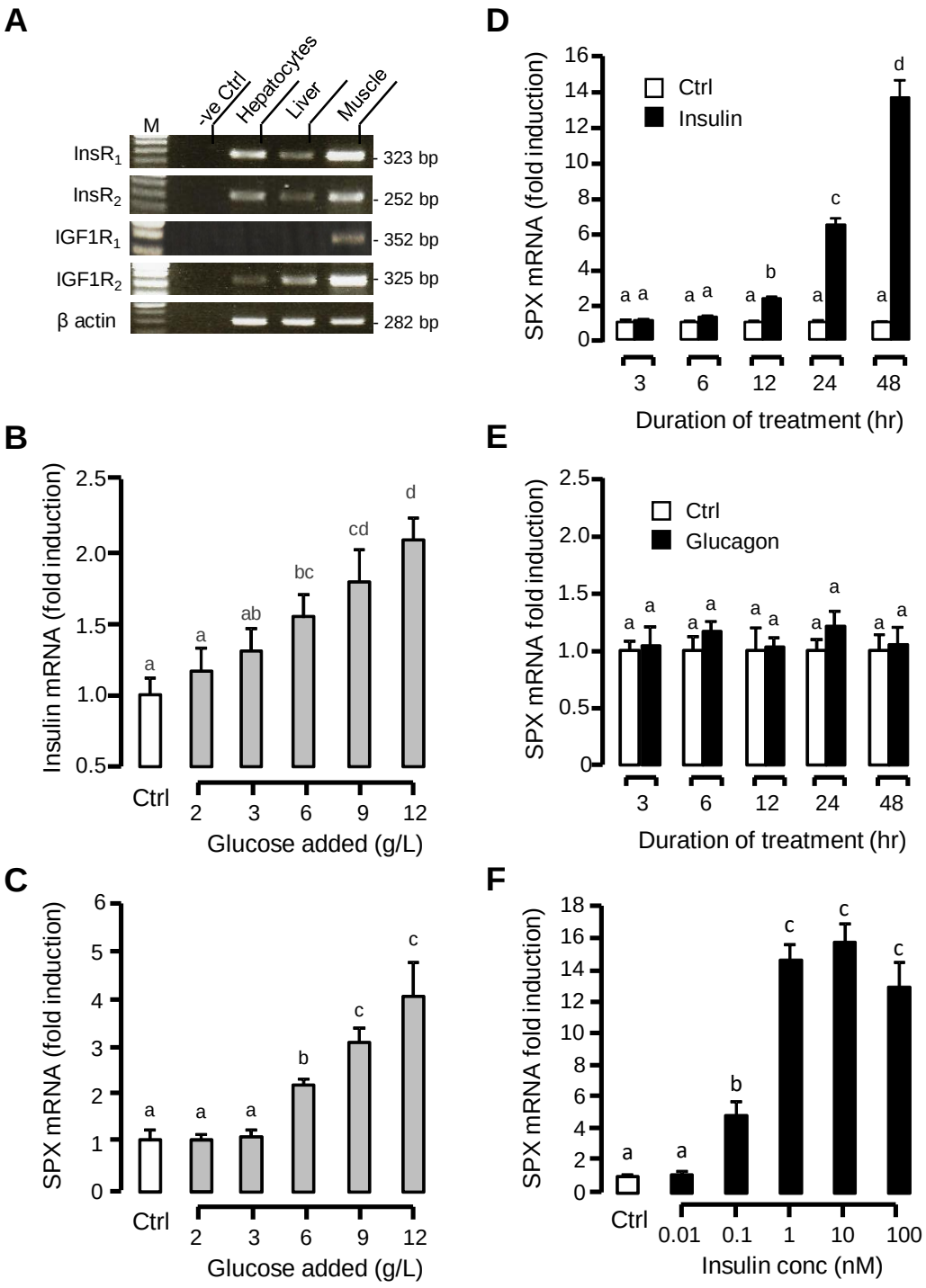
A
M
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Hepatocytes
Liver
Muscle
InsR1
InsR2
IGF1R1
IGF1R2
β actin
323 bp
252 bp
352 bp
325 bp
282 bp
B
Insulin mRNA (fold induction)
Ctrl
Glucose added (g/L)
C
SPX mRNA (fold induction)
Ctrl
Glucose added (g/L)
D
SPX mRNA (fold induction)
Ctrl
Insulin
Duration of treatment (hr)
E
SPX mRNA fold induction)
Ctrl
Glucagon
Duration of treatment (hr)
F
SPX mRNA fold induction)
Ctrl
Insulin conc (nM)

Fig.7

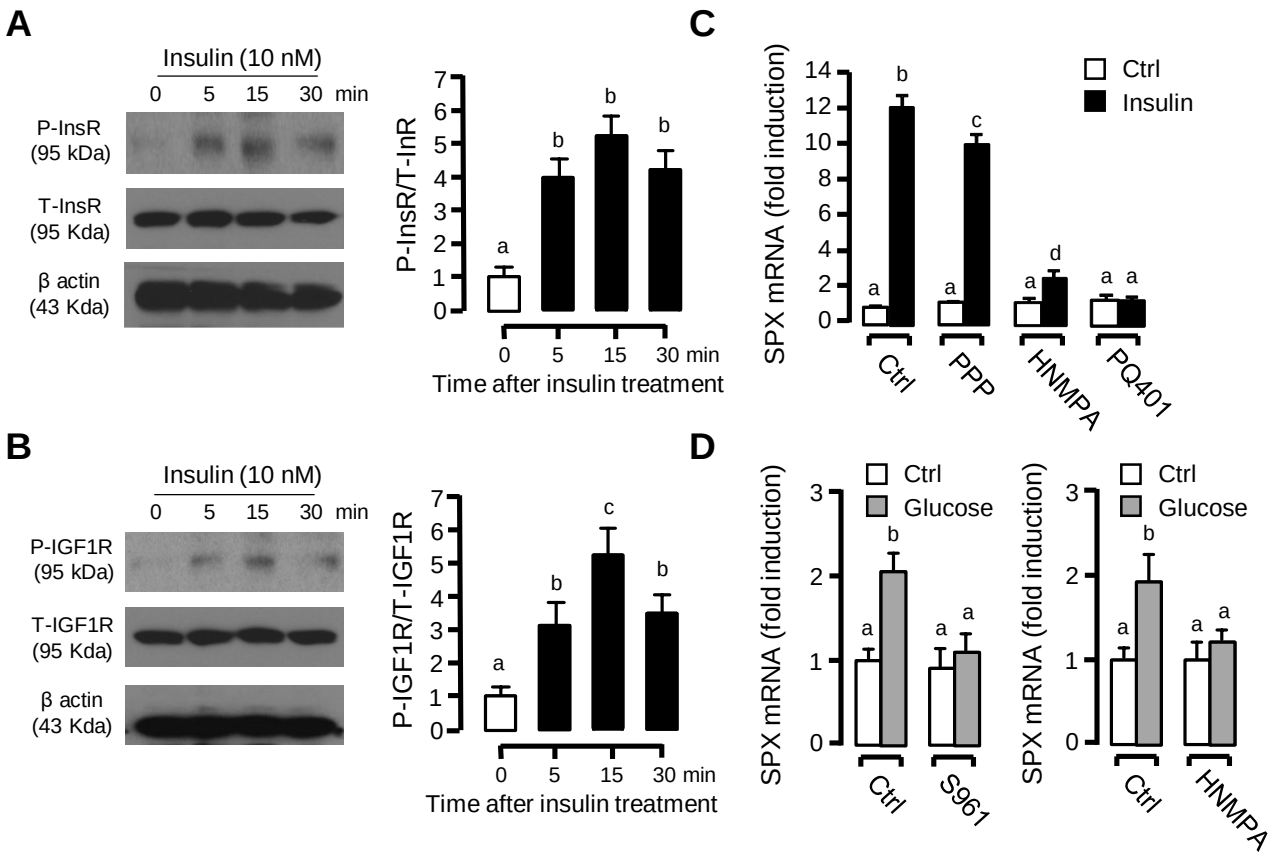


Fig.8

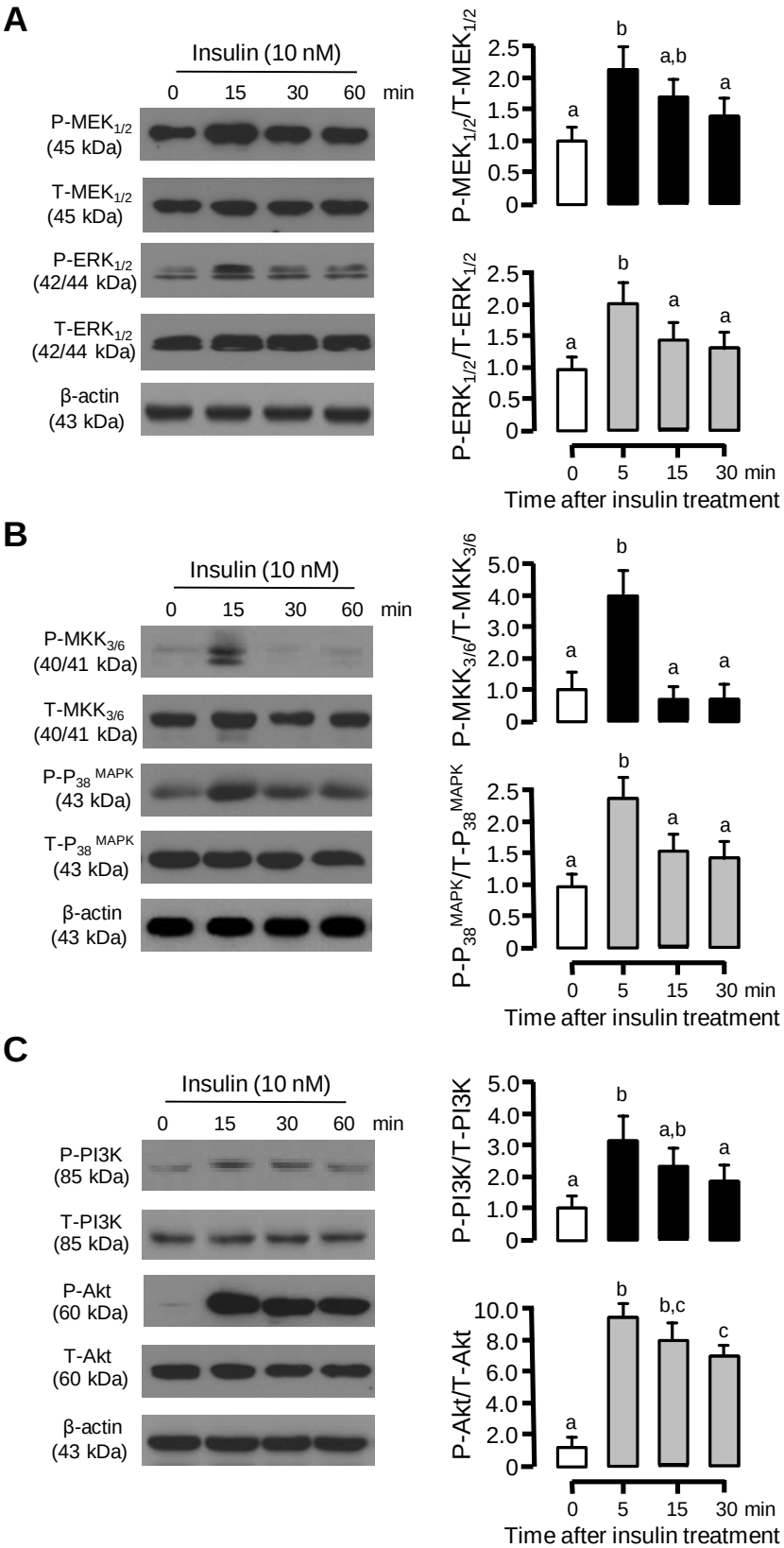


Fig.9

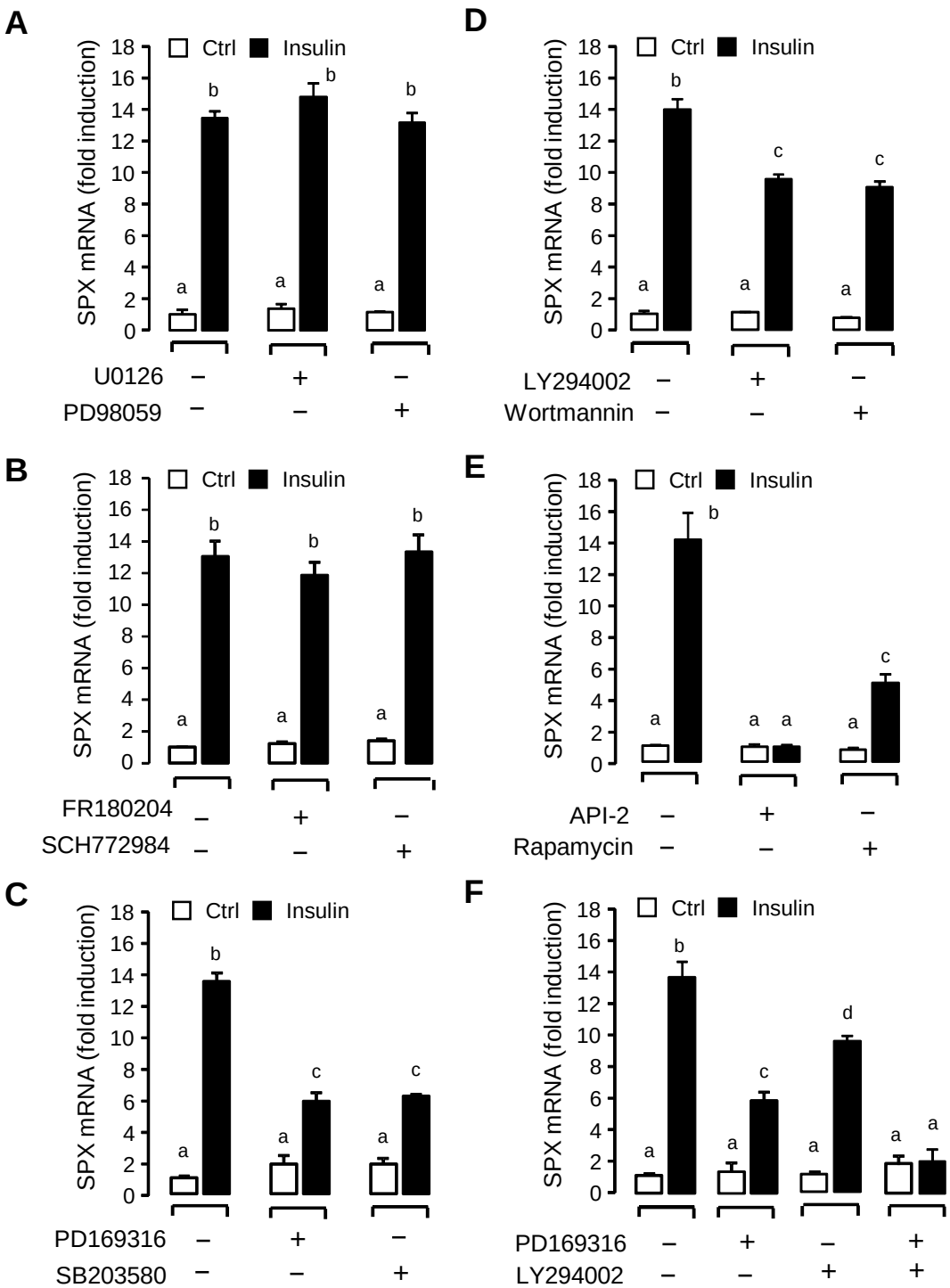


Fig.10

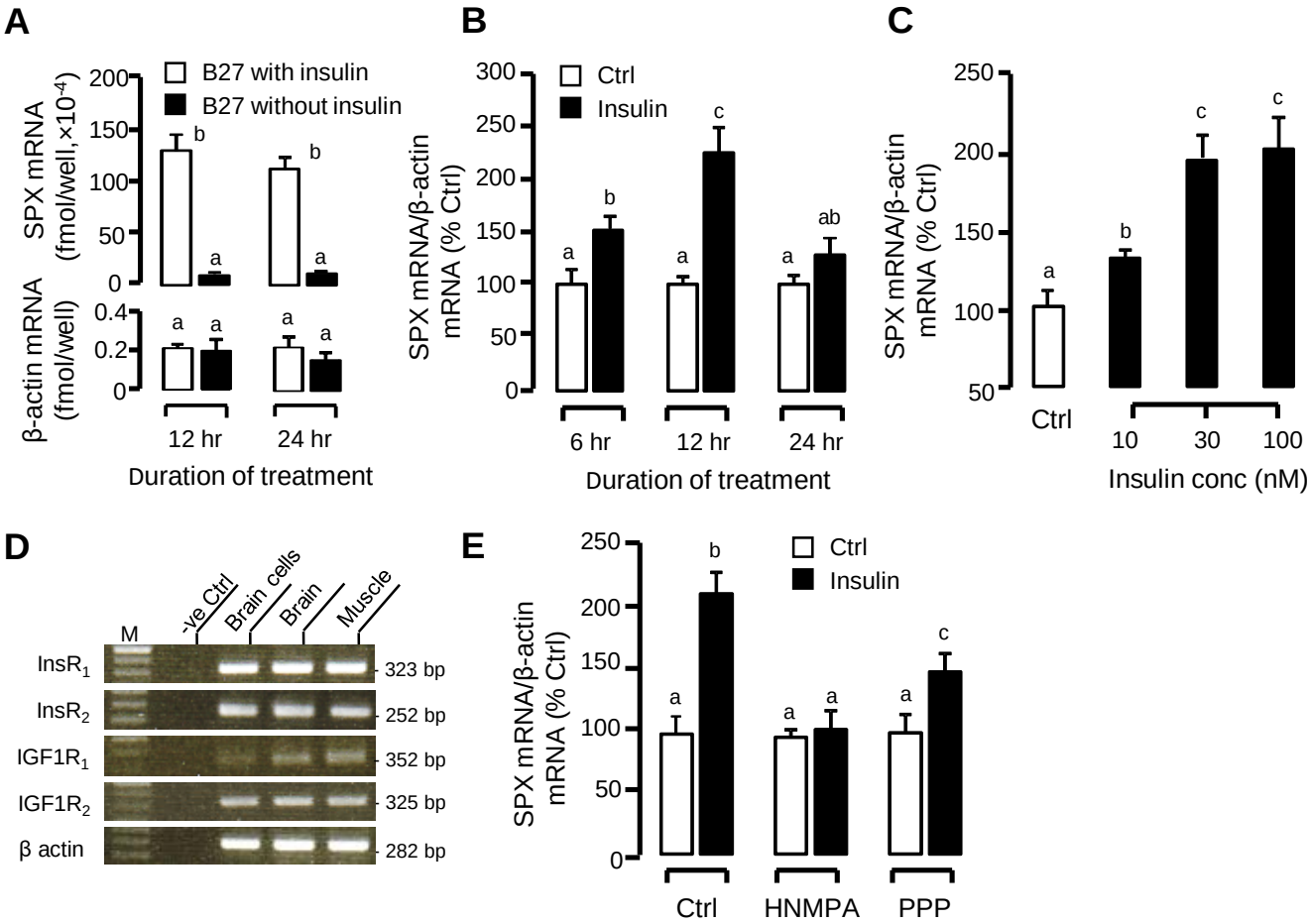


Fig.11

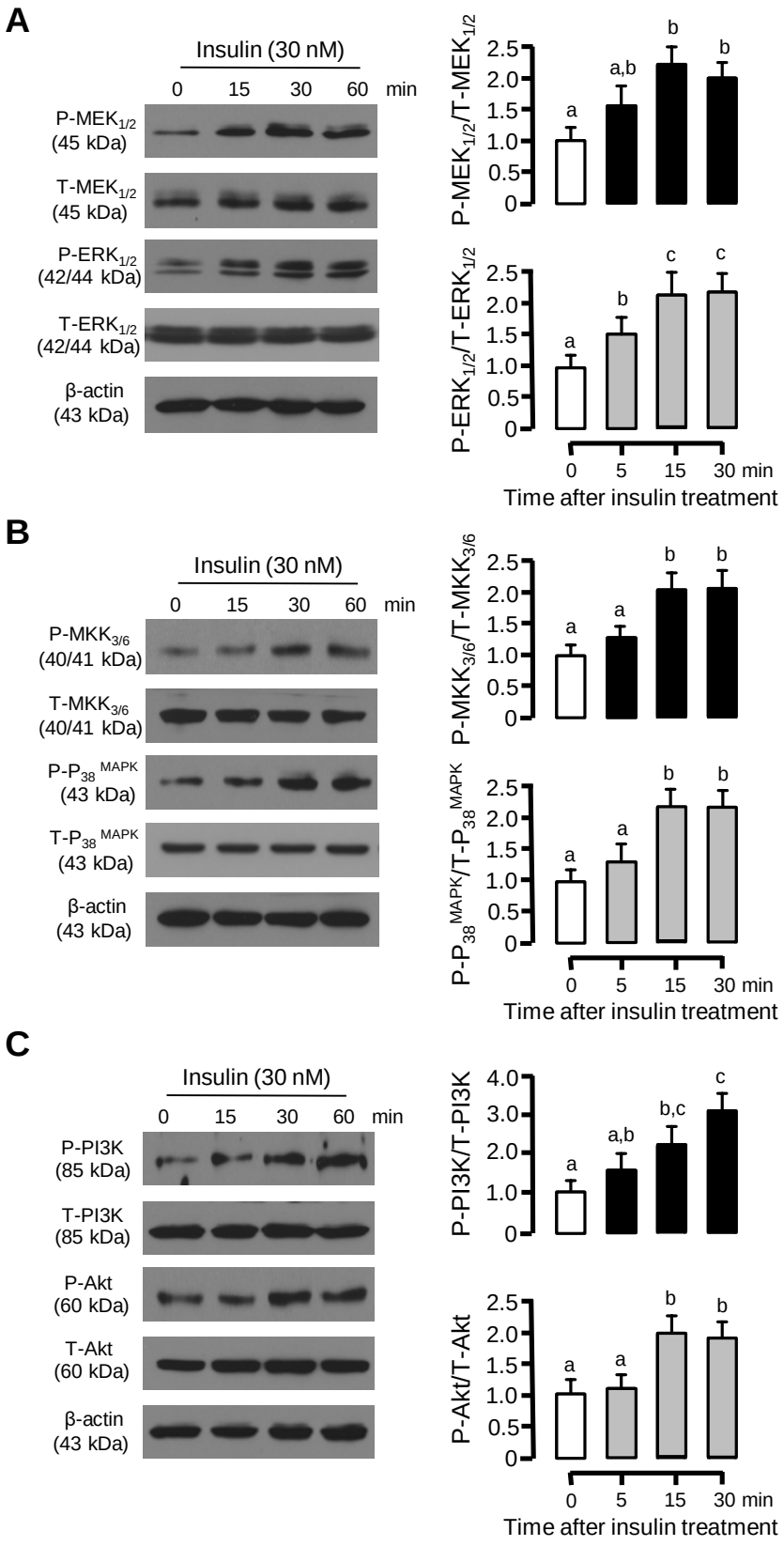
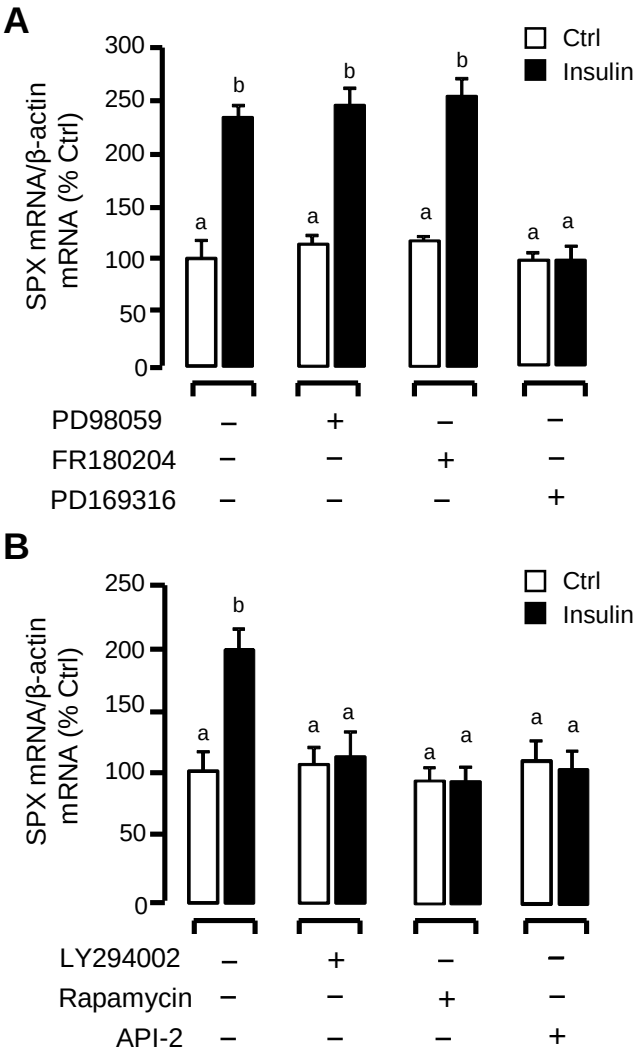


Fig.12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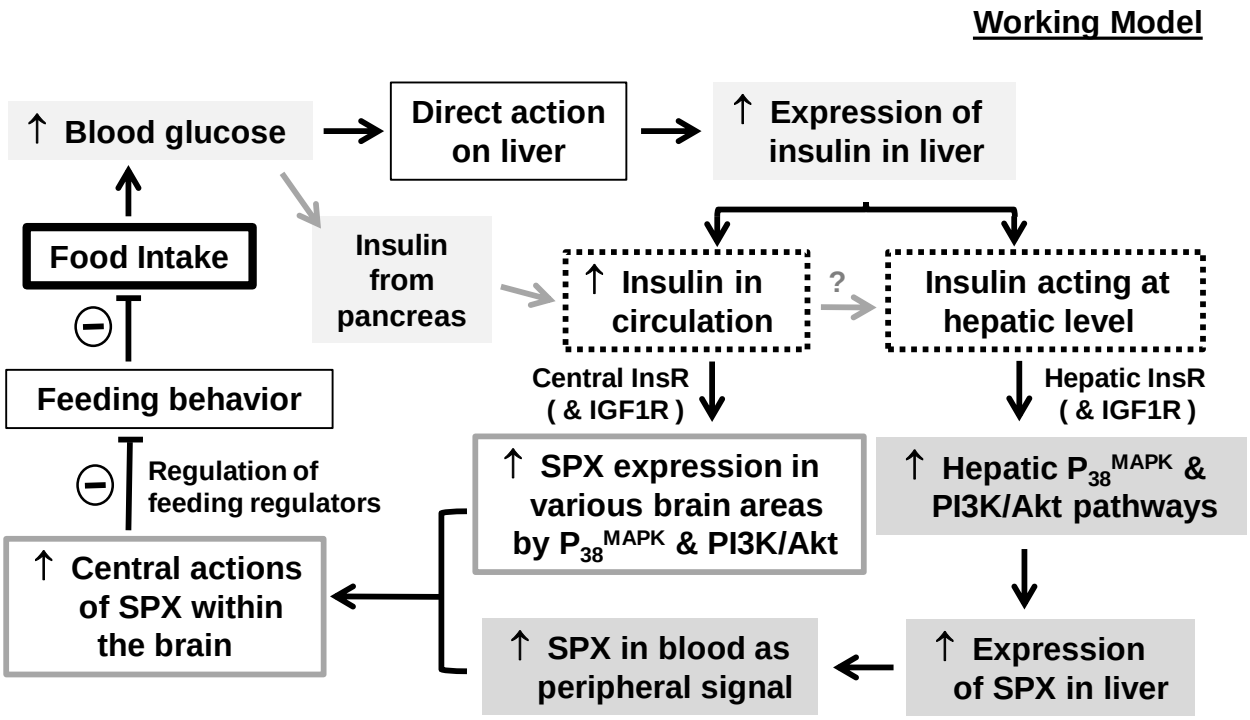
SPX mRNA/β-actin mRNA (% Ctrl)
300
250
200
150
100
50
0
Ctrl
Insulin
a
b
a
b
a
b
a
a
PD98059 – + – –
FR180204 – – + –
PD169316 – – – +
B
SPX mRNA/β-actin mRNA (% Ctrl)
250
200
150
100
50
0
Ctrl
Insulin
a
b
a
a
a
a
a
a
LY294002 – + – –
Rapamycin – – + –
API-2 – – – +

Fig.13


Working Model
↑ Blood glucose
Direct action on liver
↑ Expression of insulin in liver
Food Intake
Insulin from pancreas
↑ Insulin in circulation
?
Insulin acting at hepatic level
Feeding behavior
Central InsR (& IGF1R)
Hepatic InsR (& IGF1R)
Regulation of feeding regulators
↑ SPX expression in various brain areas by P_{38}^{MAPK} & PI3K/Akt
↑ Hepatic P_{38}^{MAPK} & PI3K/Akt pathways
↑ Central actions of SPX within the brain
↑ SPX in blood as peripheral signal
↑ Expression of SPX in liver

Supplemental Fig.01

Supplemental Fig.02

Supplemental Fig.03

Supplemental Fig.04

Antibody Table

Peptide/protein target	Antigen sequence (if known)	Name of Antibody / RRID	Manufacturer, catalog #, and/or name of individual providing the antibody	Species raised in; monoclonal or polyclonal	Dilution used
Phospho-MEK1/2	A synthetic phosphopeptide (KLP-coupled) corresponding to residues around Ser217/221 of human MEK1/2.	Phospho-MEK1/2 (Ser217/221) mAb / AB_2138017	Cell Signaling Technology, Inc., catalog #9154	monoclonal IgG in Rabbit	1:1,000 for WB
Total MEK1/2	A synthetic peptide (KLH coupled) covering the conserved region of human, rat and mouse MEK1/2.	MEK1/2 Antibody (for total MEK1/2) / AB_823567	Cell Signaling Technology, Inc., catalog #9122	polyclonal in Rabbit	1:1,000 for WB
Phospho-ERK1/2	A synthetic peptide (KLH coupled) with HTGFLTpEYpVAT sequence corresponding to the phosphorylated form of ERK-activation loop.	Diphosphorylated ERK1/2 mAb / AB_477245	Sigma-Aldrich Co. , catalog #M8159	monoclonal IgG in Mouse	1:5,000 for WB
Total ERK-1/2	A synthetic peptide (KLH coupled) with RRITVEEALAHPYLEQ YYDPTDE sequence derived from subdomain-XI of human ERK1/2.	ERK1/2 Antibody (for total ERK1/2) / AB_477216	Sigma-Aldrich Co. , catalog #M5670	polyclonal in Rabbit	1:5,000 for WB
Phospho-MKK3/6	Polyclonal antibodies are produced by immunizing animals with a synthetic phosphopeptide corresponding to residues around Ser189/207 of human MKK3	Phospho-MKK3 (Ser189)/MKK6 (Ser 207) Antibody / AB_2140799	Cell Signaling Technology, Inc., catalog #9231	polyclonal in Rabbit	1:2,000 for WB
Total MKK3	Synthetic peptide directed towards the C terminal of human MAP2K3 (EEPSPQLPADRFSPEFVDFTAQCLRKNPAERMSYLELMEHPFFTLHKTKK)	MAP2K3 (mitogen-activated protein kinase kinase 3) Antibody (against C terminal of MAP2K3)/ AB_2046667	AVIVA SYSTEMS BIOLOGY, catalog #ARP42065_P050	polyclonal in Rabbit	1:2,000 for WB
Phospho-p38 MAPK	A synthetic phosphopeptide corresponding to residues around Thr180/Tyr182 of human p38 MAPK.	Phospho-p38 MAPK (Thr180/Tyr182) Antibody / AB_331641	Cell Signaling Technology, Inc., catalog #9211	polyclonal in Rabbit	1:1,000 for WB
Total p38 MAPK	A synthetic peptide corresponding to the sequence of human p38 MAPK.	p38 MAPK Antibody /AB_330713	Cell Signaling Technology, Inc., catalog #9212	polyclonal in Rabbit	1:1,000 for WB
Phospho-PI3K	Polyclonal antibodies are produced by immunizing animals with a synthetic phosphopeptide corresponding to residues surrounding Tyr458 of mouse p85	Phospho-PI3 Kinase p85 (Tyr458)/p55 (Tyr199) Antibody / AB_659940	Cell Signaling Technology, Inc., catalog #4228	polyclonal in Rabbit	1:2,000 for WB
Total PI3K	Monoclonal antibody is produced by immunizing animals with a synthetic peptide corresponding to the sequence of human PI3K p85.	PI3 Kinase p85 (19H8) Rabbit mAb / AB_10695255	Cell Signaling Technology, Inc., catalog #4257	monoclonal IgG in Rabbit	1:2,000 for WB
Phospho-Akt	A synthetic phosphopeptide (KLH-coupled) corresponding to residues surrounding Ser473 of mouse Akt.	Phospho-Akt (Ser473) Antibody / AB_329825	Cell Signaling Technology, Inc., catalog #9271	polyclonal in Rabbit	1:5,000 for WB
Total Akt	A synthetic peptide (KLH-coupled) derived from the carboxy-terminal sequence of mouse Akt.	Akt Antibody (for total Akt) / AB_329827	Cell Signaling Technology, Inc., catalog #9272	polyclonal in Rabbit	1:1,500 for WB
Phospho-InsR	Synthetic phosphopeptide derived from human Insulin Receptor around the phosphorylation site of tyrosine 1361 (I-P-YP-T-H).	Insulin Receptor (phospho Y1361) antibody / AB_943587	Abcam, catalog #ab60946	polyclonal in Rabbit	1:2,000 for WB