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γ-Aminobutyric Acid Up-Regulates the Expression of a Novel Secretogranin-II Messenger Ribonucleic Acid in the Goldfish Pituitary*

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ABSTRACT

An RNA-arbitrarily primed PCR differential display strategy was used to identify candidate genes in the pituitary that are up-regulated by endogenously activated γ-aminobutyric acid (GABA) systems that may also be involved in the control of reproduction. Goldfish were injected with the GABA metabolism inhibitor γ-vinyl-GABA (GVG), known for its high efficiency to specifically increase endogenous brain and pituitary GABA levels in this species, resulting in higher levels of circulating gonadotropin-II (GTH-II). Several transcripts related to hormone secretion, signal transduction pathways, and messenger RNA (mRNA) editing were shown to be up-regulated after GVG injection. Among these transcripts we characterized an mRNA coding for the secretory vesicle protein secretogranin-II (SgII), a member of the chromogranin family, which is the precursor of a novel 34 amino acid neuropeptide, goldfish secretoneurin (SN). A semiquantitative PCR developed to measure pituitary SgII mRNA levels showed a 5-fold increase in GVG treated fish *vs.* control fish. Moreover, GVG treatment specifically increased SgII mRNA levels in gonadotrophs, concomitant with a decrease in GTH-II cell content. In addition, ip injection of synthetic goldfish SN increased GTH-II release in goldfish pretreated with the dopamine antagonist domperidone. Activation of GABAergic neurons has two effects, enhancing *in vivo* GTH-II release and up-regulating SgII mRNA specifically in goldfish gonadotrophs. Together with our SN bioactivity data, this suggests the existence in the pituitary of an autocrine or paracrine mechanism linked to the regulated secretory pathway in the gonadotrophs. (*Endocrinology* **139:** 4870–4880, 1998)

THE AMINO ACID γ-aminobutyric acid (GABA) is one of the most abundant neurotransmitters in the vertebrate central nervous system and is considered to be a classical inhibitory neurotransmitter inducing postsynaptic membrane hyperpolarizations. However, in addition to the predominant inhibitory actions reported for GABA, there is increasing evidence in both vertebrates (1) and invertebrates (2) that GABA also has important depolarizing and stimulatory actions. In the rat hypothalamus, for example, GABA can be found in approximately 50% of presynaptic boutons (3) and regulates most aspects of hypothalamic function. In particular, GABA via the $GABA_A$ receptor is excitatory in neonatal hypothalamic neurons, whereas in adults the opposite has been shown (4). Recently, dual hyperpolarizing and depolarizing actions of GABA have been shown in the adult rat suprachiasmatic nucleus of the hypothalamus (5). Within this nucleus, GABA is inhibitory at night but has important excitatory actions in the daytime, which may be part of the molecular mechanism of the circadian clock, coordinating diurnal changes in behavior and physiology.

In adult vertebrates, GABAergic control of neuroendocrine function is also believed by many to be mainly inhibitory. However, a significant stimulatory role for GABA in the control of hypothalamic GnRH (GnRH) (6) and pituitary gonadotropic hormone (7, 8) release is now apparent. Our work using the adult goldfish model (reviewed in Ref. 9) has demonstrated that GABA has a clear and potent stimulatory effect on pituitary gonadotropin-II (GTH-II; the fish homolog of LH), which in turn stimulates reproductive function (*i.e.* gonadal sex steroid production, ovulation, or sperm release). The goldfish, as in other bony fish, lack a hypothalamo-pituitary portal system and the proximal pars distalis, that part of the anterior pituitary containing the gonadotroph and somatotroph cells, is directly innervated by a multitude of neurons synthesizing neuropeptides and classical neurotransmitters (10), including GABA (11). Moreover, because nerve terminals reside within the anterior pituitary complex, this is a unique system in which to study neurotransmitter-endocrine cell interactions (10).

Significantly, the magnitude of the GTH-II secretory response is often greater to GABA than to any other peptide or neurotransmitter acting on the system, indicating that GABA is a pivotal neurotransmitter for central reproductive control. *In vivo*, pharmacological evidence indicates that GABA action to enhance GTH-II release is mediated by dual stimulatory effects on $GABA_A$ and $GABA_B$ receptors (8), which may explain why GABA has a dominant stimulatory effect in the goldfish model. GABA acts to stimulate GTH-II release

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by enhancement of GnRH release from the nerve terminal within the goldfish pituitary and also by inhibition of the inhibitory preoptic-hypothalamic dopaminergic system. We have also demonstrated that GABA-stimulated GTH-II release is a physiologically relevant signal leading to enhanced gonadal steroid production (12). Sex steroids in turn modulate GABA responses; testosterone increases, whereas estradiol decreases GABA-induced GTH-II release *in vivo*. Both steroids also modulate GABA synthesis in the goldfish preoptic-hypothalamic region and pituitary (8). The role of GABA in stimulating GnRH and GTH-II release, and the exquisite sensitivity of the GABA system to sex steroid feedback, underscores the significance of this neurotransmitter in the central regulation of reproduction.

To identify candidate genes in the pituitary that are upregulated by endogenously activated GABA systems that may also be involved in controlling GTH-II release, we have used an RNA-arbitrarily primed PCR (RAP-PCR) differential display strategy. Injection of the GABA metabolism inhibitor γ-vinyl-GABA (GVG), also known as Vigabatrin, a widely prescribed antiepileptic (13) increases endogenous brain and pituitary GABA levels. Specifically, the effects of GVG to elevate endogenous GABA levels have been previously validated in the goldfish (6, 12). We isolated and identified several pituitary genes related to hormone secretion, signal transduction pathways, and messenger RNA (mRNA) editing that were differentially increased following GVG treatments. Here we characterize one major product, the secretory vesicle protein secretogranin-II (SgII), a member of the chromogranin superfamily, which is the precursor of a novel bioactive neuropeptide, goldfish secretoneurin (SN). In rats, SN is coreleased with LH after GnRH stimulation (14). In addition, SgII has been proposed to be involved in packaging of LH into the secretory vesicles (15), further implicating this molecule in the regulation of endocrine cell function. Our results show that associated with GABA-stimulated GTH-II release is the increased expression of SgII mRNA in the gonadotrophs. We also show for the first time that synthetic goldfish secretoneurin has significant bioactivity and rapidly increases GTH-II release *in vivo*, suggesting the existence of an autocrine or paracrine mechanism within the pituitary.

Materials and Methods

Animals and rearing conditions

Common goldfish (*Carassius auratus*) were purchased throughout the year from commercial suppliers (Mount Parnell, PA, and Grassyforks, MO). Fish were acclimated to 18 C under a natural simulated photoperiod and fed and maintained on standard flaked goldfish food. Before any handling for drug injection, blood sampling and tissue collections, fish were lightly anesthetized by immersion in 0.05% tricaine methane sulfonate (TMS). Typically, recovery from TMS is rapid, taking less than 5 min. Blood samples were taken by puncture of the caudal vasculature using a 25 gauge needle attached to a 1-ml syringe. Blood was allowed to clot for 16–24 h and serum collected by centrifugation. At the termination of an experiment, fish were killed and the gonadosomatic index (GSI = gonad weight/body weight × 100) calculated as an indication of stage of the seasonal reproductive cycle.

Effect of GVG on serum GtH-II levels

Our previous studies have characterized the timecourse, specificity, and use of D, L-γ-vinyl GABA (D, L-4-amino-hex-5-enoic-acid; GVG or Vigabatrin) to inhibit the GABA metabolism enzyme, GABA transaminase, in goldfish brain (6, 12). Following a single injection of GVG in goldfish, endogenous hypothalamic and pituitary GABA levels increase approximately 3-fold, leading to increased serum GTH-II levels within 8 h and for up to several weeks (8, 12). At different times during the seasonal reproductive cycle, GVG was dissolved in 0.6% saline (NaCl) vehicle and ip injected in 10 μl/g body weight at a final concentration of 300 μg/g body weight. Control groups received equivalent volumes of the vehicle. Blood from the different groups was collected by caudal puncture 24 h after injections. Serum GTH-II levels were measured using a specific double antibody RIA (16)

RNA isolation and RT

Pituitaries or brain were rapidly dissected and frozen on dry ice or in liquid nitrogen for subsequent isolation of total or poly(A)$^+$ RNA as required. For total RNA isolation, tissues were sonicated in RNAzol (Biogenesis, Poole, UK), isopropanol-precipitated and washed in 75% ethanol. Poly(A)$^+$ RNA was purified from total RNA pools (Oligotex mRNA mini kit, Quiagen Ltd., Crawley, UK) or directly from tissues (Quick prep micro mRNA purification kit, Pharmacia Biotech, St. Albans, UK). Complementary DNA (cDNA) was obtained from either 100 ng of poly(A)$^+$ RNA or 1 μg of total RNA using MMLV reverse transcriptase and oligo dT primer (Promega Corp., Madison, WI). Ten to 15 pituitaries were pooled to obtain sufficient total RNA.

Differential display and transcript identification

GVG regulated transcripts were identified by differential display (17) using the RAP-PCR kit (Stratagene, La Jolla, CA). Briefly, poly(A)$^+$ RNA was reversed transcribed to cDNA using a series of single18-base arbitrary primers (primer A_1: 5′-AATCTAGAGCTCCTCCTC-3′; primer A_2: 5′-AATCTAGAGCTCCAGCAG-3′) in both first and second strand cDNA synthesis. The resulting cDNAs were subsequently PCR-amplified in the presence of [α^{32}P]dATP and the same arbitrary primer used to synthesize the first strand. Briefly, the PCR reaction consisted of a single cycle of low-stringency amplification (annealing temperature 36 C) followed by 40 cycles with higher stringency (annealing temperature 54 C). PCR products were loaded onto a 4% acrylamide/7 M urea sequencing gel and electrophoresed at 1200v for 4 h. Gels were dried and exposed to Blue sensitive x-ray film (Genetic Research Instrumentation Ltd., Dunmow, UK) for 48 h at −70 C. A control reaction to test the quality of the cDNA was performed using oligo(dT_{18}) primer instead of the arbitrary primers for the cDNA first strand. To confirm the efficiency of RT reactions, the cDNAs were also amplified using β-actin primers. To eliminate false positives a 1/10th dilution of the original template was also used to perform the RAP-PCR (17). The reactions were also repeated at least four times to confirm the accuracy of the banding patterns obtained before isolation and subsequent cloning for the cDNA fragments. Differentially expressed transcripts > 400 bp in length demonstrating up-regulation by GVG were then carefully cut from the gel and the DNA was extracted in TE buffer, cloned into a pCR II vector, and transformed in *Escherichia coli* competent cells using the TA-cloning kit (In vitrogen BV, Leek, The Netherlands). White colonies were selected from X-Gal ampicillin LB agar plates and grown in LB liquid media. Plasmids were prepared and purified using the Quiagen Mini Kit. Cloned inserts were sequenced using an ABI 377 automated sequencer (PE Applied Biosystems; Warrington, UK) and submitted to FASTA for comparison to known sequences accessible in GenBank/EMBL.

Northern blot analysis

Total RNA (30 μg) or poly(A)$^+$ RNA (6 μg) was electrophoresed on 1.2% agarose/formaldehyde gels, transferred onto nylon membranes (Hybond N$^+$, Amersham International Ltd., Little Chalfont, UK) by vacuum blotting and fixed at 80 C for 90 min. Membranes were prehybridized in Rapid-Hyb buffer (Amersham International Ltd.) at 65 C for 30 min. Hybridizations to [α^{32}P]dATP random labeled (MegaPrime, Amersham International Ltd.) SgII and β-actin cDNA probes were carried out under standard conditions at 65 C for 4 h. After hybridization, membranes were sequentially washed to high stringency (0.1 × SSC/0.1% SDS at 65 C), and specific hybridization signals were visualized autoradiographically at −80 C for 48 h using two intensifying screens.

To control for RNA loading, the membranes were stripped with boiling 0.1% SDS and hybridized with radiolabeled goldfish β-actin cDNA as described above. This probe was used as internal standard and its expression was not affected by GVG injections. To assess the size of the obtained transcripts an RNA marker (0.2–10 kb, R&D Systems Europe Ltd., Abingdon, UK) was electrophoresed together with the RNA samples.

Semiquantitative RT-PCR analysis of mRNA transcripts

One hundred nanograms of total RNA were reverse transcribed to cDNA and subjected to PCR amplification in the presence of [α^{32}P]dATP. Samples were withdrawn every 5 cycles (ranging from 10 to 40 cycles) to determine amplification kinetics of the reaction and products were separated on 8% acrylamide gels. Gels were dried and the reaction products visualized by autoradiography at −80 C for 6–12 h. Radioactive bands were excised, extracted in 0.5 N quaternary ammonium hydroxide in toluene (Soluene-350; Packard Instrument Co., Inc.) and measured by liquid scintillation counting. Primers used for SgII were 5′-TTCTTACCACGCTACAACAG-3′ and 5′-TCATCATCTTCGCCATCCTC-3′; and for β-actin 5′-GAGACCTTCAACACCCC-3′ and 5′-CCAAGAAGGATGGCTGGA-3′. Conditions were validated for both SgII and β-actin (denaturation at 95 C for 1 min, annealing at 54 C for 2 min, and extension at 72 C for 2 min). To reduce variability the same PCR mastermix of reagents (except for the primers) was used for both SgII and β-actin. As PCR conditions for both molecules were exactly the same, amplifications were performed at the same time. For every PCR reaction, samples were run in triplicate.

cDNA library screening and sequencing

A goldfish cDNA brain plus pituitary library constructed using the ZAP Express cDNA synthesis kit (Stratagene) was obtained from Drs. K. L. Yu and M. L. He (Department of Zoology, Universty of Hong Kong). The library was screened with a goldfish cDNA SgII probe (480 bp). Libraries were plated at a density of 2 plaques per cm^2 and lifts taken in duplicate onto nitrocellulose membranes to identify false positives. The membranes were subsequently denatured (1.5 M NaCl/0.5 M NaOH; 2 min), neutralized (0.5 M Tris-HCl (pH 8.0)/1.5 M NaCl; 5 min), and washed in 2 × SSC for 1 min, and the DNA fixed by baking at 80 C for 2 h. Probe preparation and hybridizations were as described for Northern blot except that the membranes were hybridized for 2 h. Positive clones were identified, isolated and *in vivo* excised from the ZAP express vector with the ExAssist helper phage (Stratagene) to generate subclones in the pBK-CMV phagemid vector. The vector was transformed in XLOLR *Escherichia coli* strain (kanamycin resistant) and positive colonies were selected from kanamycin LB agar plates and checked with PCR for the presence of the correct insert. Plasmids were purified and the cDNA inserts sequenced. Two different clones were sequenced in both forward and reverse directions to obtain the nucleotide sequence reported. Phylogenetic analysis of SgII based on DNA distances (Fitch, Kitsch and Neighbor-Joining methods), maximum likelihood, or parsimony were carried out using the PHYLIP statistical package in GCG.

Isolation of gonadotrophs and somatotrophs

To determine the pituitary cell type responding to GVG with an increase in SgII expression, pituitaries were trypsin dispersed as described and validated for goldfish (18). Briefly, pituitaries from large (80–200 g) spermiating male goldfish (n = 80) previously injected with saline or GVG were collected under sterile conditions, washed in dispersion media, carefully fragmented, and subjected to standard mild trypsinization and dispersion following gentle agitation. After dispersion, the different cell types were isolated in a Percoll centrifugation gradient and cell viability determined by hemocytometry (18). Six different fractions were obtained and fractions 5 and 6, containing mostly gonadotrophs, were pooled. Cell contents of GTH-II (16) and GH (19) were determined by specific RIAs. Cells were concentrated by centrifugation, washed, and resuspended in culture medium and rapidly frozen on dry ice. Poly(A)$^+$ RNA was directly isolated (Quick prep micro mRNA purification kit, Pharmacia Biotech) from the fractions for cDNA synthesis. From each cell fraction two separate pools of cDNA were obtained from independent RT reactions and used to perform a semiquantitative PCR for SgII and β-actin as previously described.

Synthesis of goldfish SN and in vivo bioactivity studies

Based on the predicted amino acid sequence and conserved dibasic cleavage sites, goldfish secretoneurin, consisting of 34 amino acids, was synthesized using a continuous flow Fmoc-based peptide synthesis protocol on a Pioneer peptide synthesizer and subsequently purified to > 95% by HPLC. The HPLC solvent system consisted of 50 mM diisopropylethyl ammonium acteate (pH 5.7) and SN was eluted on a Poros R_2 column (PerSeptive Biosystems, Framington, MA; 4.6 mm × 100 mm) with 70% acetonitrile in water (flow rate = 1 ml/min; gradient 15–100% over 30 min). The, molecular weight (3656) of the SN peptide was confirmed on a matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometer.

Previous studies in goldfish have demonstrated that GTH-II release *in vivo* is under a potent, tonic inhibition by dopamine (20). In fact, in sexually regressed fish, the hypothalamic neuropeptide GnRH is ineffective in stimulating GTH-II release unless DA inhibition is reduced by pretreatments with DA antagonists or DA synthesis inhibitors (9, 20). Therefore, the effects of SN were tested in sexually regressed fish injected ip with the DA receptor antagonist domperidone (10 μg/g BW) in DMSO vehicle (1 μl/g BW) 24 h before experimentation. Controls received an equivalent volume of DMSO. Domperidone is specific for the DA_2 receptor and does not cross the goldfish blood-brain barrier (21). The following day, fish were injected ip with synthetic goldfish SN (1 μg/g BW) or 0.6% saline vehicle (1 μl/g BW; pH 6.4). Blood was collected 30 min after SN injection. The dose of SN was chosen to produce levels in the range of SN concentrations found in the rat median eminence (22) and anterior pituitary (23). Based on the relatively inefficient uptake of ip injected protein hormones (24) and short half-life (30–40 min) of iv injected neuropeptides (25) as established in goldfish, we estimate that levels of SN in the goldfish pituitary following ip injection of 1 μg/g BW would produce increases in the nmol range, in line with levels produced by rat pituitary tissues, and within the dose range of bioactivity studies on DA release from rat brain (26).

Statistical analysis of data

Unpaired t test or one-way ANOVA (Multistat Package; Biosoft Inc., Ferguson, MO) were used as appropriate to test for statistical differences between groups. Group means were considered different if $P < 0.05$.

Results

RAP-PCR

The differential display strategy using RAP-PCR was designed to identify GVG regulated transcripts in goldfish pituitary. Sequence analysis revealed that several genes related to hormone secretion, signal transduction, and RNA processing were up-regulated by GVG treatments (Table 1). One of the differentially displayed gene transcripts was secretogranin II, also known as chromogranin C, the first chromogranin isolated in fish. Other up-regulated transcripts identified were goldfish homologs of human GTPase activating protein, human FKBP12-rapamycin associated protein, human arginine-rich nuclear protein, and human polypirimidine tract binding protein. One band that was not differentially regulated was selected as an additional control for the RAP-PCR reaction. This was identified as the goldfish homolog of a mouse phosphatidylinositol 3-kinase (PI-3K), p85α and was not affected by GVG treatments in the pituitary. In addition, PI-3K was also found to be highly expressed in brain (not shown) and, as we observed in the pituitary, its expression was not affected by GVG injections. Although our focus here has been on up-regulated products,

TABLE 1. GABA-regulated genes in the goldfish pituitary as determined using RAP-PCR

Molecule	Functions	Fragment length	% Identity[a]	Goldfish sequence
Secretogranin II	Secretory vesicle formation Neuropeptide precursor	582 bp	31% (Rat; P10362)	AF046002[b]
GTPase activating protein	Exocytosis Cell differentiation	537 bp	70% (Human; M64788)	AF046003
FKBP12-Rapamycin associated protein	P70-(S6)-kinase Ca^{+2} signaling Apoptosis G1 cell cycle modulation	785 bp	79% (Human; L34075)	AF046004
[c]Phosphatidylinositol 3-kinase (p85α subunit)	Inositol metabolism Growth factor action Secretory vesicle formation	534 bp	28% (Mouse; P42356)	AF046005
Arginine-rich nuclear protein	Transcription mRNA processing	543 bp	58% (Human; M74002)	AF046006
Polypirimidine tract binding protein	Initiation of translation mRNA splicing	535 bp	66% (Human; X65371)	AF046007

[a] Shown in *parentheses* is the species and accession number of the GenBank/EMBL sequence.
[b] Entire Secretogranin II sequence (2253 bp long).
[c] Not affected by GVG treatment.

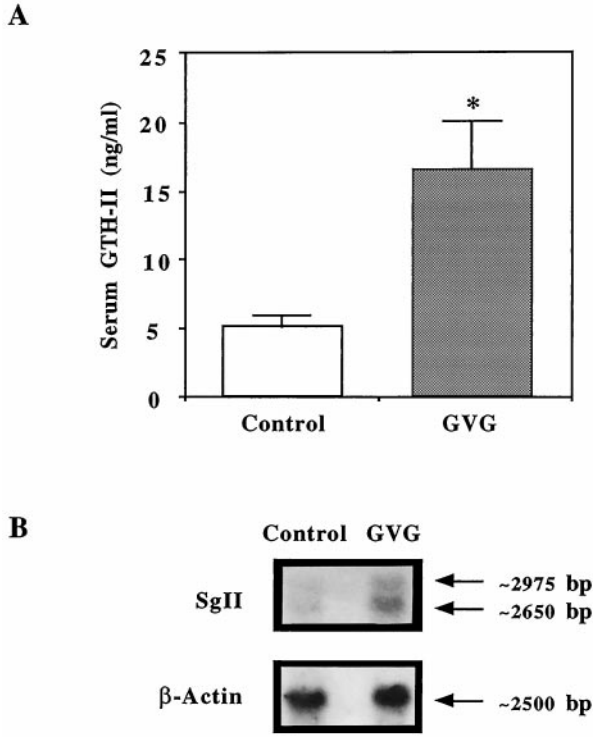

FIG. 1. Effect of GVG on serum GTH-II levels and pituitary SgII mRNA levels in goldfish. A, Goldfish in postspawning condition (GSI = 1.7 ± 0.3%) in April–May were injected ip with γ-vinyl GABA (GVG; 300 μg/g BW) or saline vehicle (control; 10 μl/g BW) and blood collected by caudal puncture 24 h later for serum GTH-II determination (n = 9 in control and n = 8 in GVG). B, Northern blot analysis of SgII in goldfish pituitary. Total RNA (30 μg) extracted from pools of pituitary glands (n = 10) was electrophoresed on a 1.2% agarose/formaldehyde gel, transferred to a nylon membrane, and hybridized with a random primed ^{32}P-labeled probe for SgII. As a control for loading, the RNA blot was stripped and rehybridized with a goldfish β-actin cDNA probe. *Arrows* indicate approximate sizes of the transcripts

GVG also decreased the expression of several gene transcripts. These have not been characterized and await further study.

Northern blot analysis

Injection of GVG stimulated GTH-II release in postspawning goldfish (Fig. 1A). A Northern blot hybridization was performed to determine the size of SgII mRNA and also to confirm that its expression is up-regulated by GVG, as previously found in the differential display analysis. Two different SgII transcripts of approximately 2975 bp and 2650 bp were expressed in goldfish pituitary and both were up-regulated by GVG. In contrast, β-actin mRNA was unaffected by GVG treatment (Fig. 1B). SgII was also highly expressed in goldfish brain and as shown in the pituitary, two transcripts were also evident in the telencephalon-hypothalamus region (Fig. 2).

cDNA library screening

The screening of the goldfish brain-pituitary cDNA library resulted in the isolation of a cDNA clone of 2253 bp long (data not shown). It contains an open reading frame of 1806 bp coding for goldfish SgII and is flanked by noncoding regions at both ends. Presented in Fig. 3 is the entire goldfish SgII deduced amino acid sequence beginning with a methionine (ATG) in the signal peptide. A search of GenBank databases showed that the protein shares an overall homology (44% similarity) with other known SgII molecules only. Alignment of the deduced amino acid sequence of goldfish SgII with SgIIs from other species indicates that only 6 dibasic sites (numbered from 1 to 6) appear to be conserved from fish to mammals (Fig. 3). However, although sites 1 and 4 are present in goldfish, they appear to be displaced 3 and 2 amino acids respectively toward the C-terminal end of SgII. The last dibasic cleavage site (number 7; Fig. 3) flanking a potential bioactive peptide known as LA-42 (27) originating from the C-terminal portion of SgII does not seem to be conserved in goldfish, although a monobasic site is evident. Consistent with what is known of other SgII molecules and meeting the criteria for the sulfation consensus motif (28), a potential tyrosine sulfation site was identified on Tyr^{123} in goldfish SgII. Significantly, only the region coding for secretoneurin, one of the putative bioactive peptides (15), was conserved. Goldfish SN is 34 amino acids long, whereas

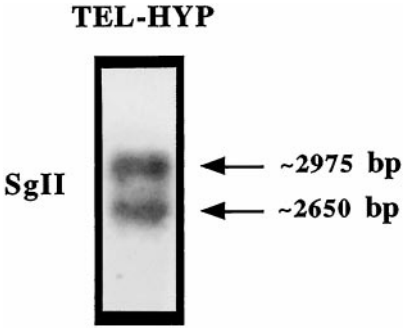

FIG. 2. Expression of SgII in the goldfish brain (telencephalon + hypothalamus). Northern blot analysis of SgII in neuroendocrine regions of the goldfish brain (telencephalon + hypothalamus). Poly(A)$^+$ RNA (6 μg) extracted from pools of brains (n = 5) was electrophoresed on a 1.2% agarose/formaldehyde gel, transferred to a nylon membrane, and hybridized with a random primed ^{32}P-labeled probe for SgII. *Arrows* indicate approximate sizes of the transcripts.

mammalian and frog SN are 33 amino acids long. Goldfish SN is 59% and 62% identical to human and Rana SN respectively, and shares a 76.5% similarity with all the species available so far. Outside the SN region, either between the N-terminal end and dibasic cleavage site 2 or between dibasic cleavage site 3 and the C-terminal end (Fig. 3), the homologies between goldfish SgII and other known SgIIs become very low (10% identical and 40% similar in both cases). In addition, phylogenetic analysis based on DNA distances (Fitch, Kitsch and Neighbor-Joining), maximum likelihood, or parsimony (PHYLIP statistical package) gave similar topologies indicating in all cases that goldfish SgII is significantly different from the known SgII sequences accessible through GenBank/EMBL (Fig. 4).

Quantitation of SgII mRNA by RT-PCR

A semiquantitative PCR protocol was developed to quantify the effects of GVG on SgII mRNA expression in the pituitary. Identical PCR conditions were used to amplify fragments of SgII (380 bp) and the β-actin (430 bp) control. When the reaction conditions are such that the amplifications remain in the exponential phase, semiquantitative analysis can be performed (29). As shown in Fig. 5, for both molecules signal amplification was linear between 10 and 25 cycles ($r^2 = 0.98$ for SgII and $r^2 = 0.95$ for β-actin; n = 6), after which product formation began to plateau. For quantitative purposes, the levels of SgII in control and GVG treated groups were standardized against the levels of β-actin amplified from the same reverse transcribed template. At 20 cycles, within the linear range of product formation, approximately 25 and 2 percent of total product yields were obtained for SgII and β-actin, respectively (Fig. 5). Injection of sexually regressed goldfish with GVG stimulated GTH-II release *in vivo* (Fig. 6A). Quantitation at 20 cycles indicates that GVG injections induced a significant ($P < 0.01$) 5-fold increase in the expression of SgII mRNA in goldfish pituitary when compared with control fish (Fig. 6, B and C). Expression of β-actin was unchanged by GVG injection.

SgII mRNA expression in gonadotrophs and somatotrophs

Percoll density gradient centrifugation was performed to isolate enriched somatotroph and gonadotroph cell fractions (18). Preparations were done in duplicate, yielding approximately 8×10^7 cells in total. The percentages of cells in the different fractions were not affected by *in vivo* GVG treatment (Fig. 7A). As a marker for each of the two cell types, GH (Fig. 7B) and GTH-II cell contents (Fig. 7C) were measured by specific RIA. GH content was increased 200% in fraction 4 only of GVG treated pituitaries (Fig. 7B). In addition, GVG treatment induced a 60% decrease of GTH-II content in fractions 5 and 6 (Fig. 7C). Semiquantitative PCR for SgII (Fig. 8, A and B) showed that GVG injection induced a 250% increase of SgII mRNA levels in gonadotroph-enriched cell fractions 5 + 6 but had no effect in somatotroph-enriched cell fraction 4.

Bioactivity of goldfish SN in vivo

Shown in Fig. 9 are the effects of SN and domperidone on GTH-II release *in vivo*. There is no sexual dimorphism in serum GTH-II levels in sexually inactive goldfish and therefore pooled values (n = 22–23) for both sexes are presented. As previously reported (20), the DA antagonist domperidone increased serum GTH-II levels by approximately 7-fold 24 h after treatment. When administered alone, SN had a slight but nonsignificant effect to increase serum GTH-II levels. However, SN significantly potentiated GTH-II release in domperidone-treated animals, additionally increasing serum GTH-II another 60% within 30 min of ip injection.

Discussion

We demonstrate for the first time that GABA has important stimulatory effects on the gene expression in an adult vertebrate pituitary. The amino acid GABA has multiple actions, regulating neurotransmission and neuroendocrine function. Although its inhibitory hyperpolarizing actions are well studied at the electrophysiological level, it is now apparent that GABA can also have important stimulatory functions in several systems. Nevertheless, the role this major neurotransmitter plays in regulating gene expression has not been previously studied in detail.

Several differentially expressed mRNA transcripts from the goldfish neuroendocrine system were isolated. The major goal of this strategy was to characterize the molecular mechanisms underlying a nonclassical stimulatory pathway activated by the inhibitory neurotransmitter GABA. A previous approach involving subtractive hybridization identified candidate genes involved in neuronal plasticity after kainate (a glutamate agonist) injections in the rat hippocampus (30). However, although this has proved to be extremely useful, construction of cDNA libraries and subtractive cDNA cloning is time consuming. In addition, our interest was specifically to identify genes potentially regulated by endogenous GABA release rather than exogenous administration of agonists. Thus, we used RAP-PCR to preferentially identify upregulated genes following treatment with GVG, a GABA-transaminase inhibitor characterized as an antiepileptic in humans (13), which has previously been demonstrated to be highly effective in elevating endogenous GABA levels and stimulating hypothalamic-pituitary function in goldfish (8, 12).

Our focus here has been on one major RAP-PCR product, the newest member of the secretogranin family, goldfish SgII.

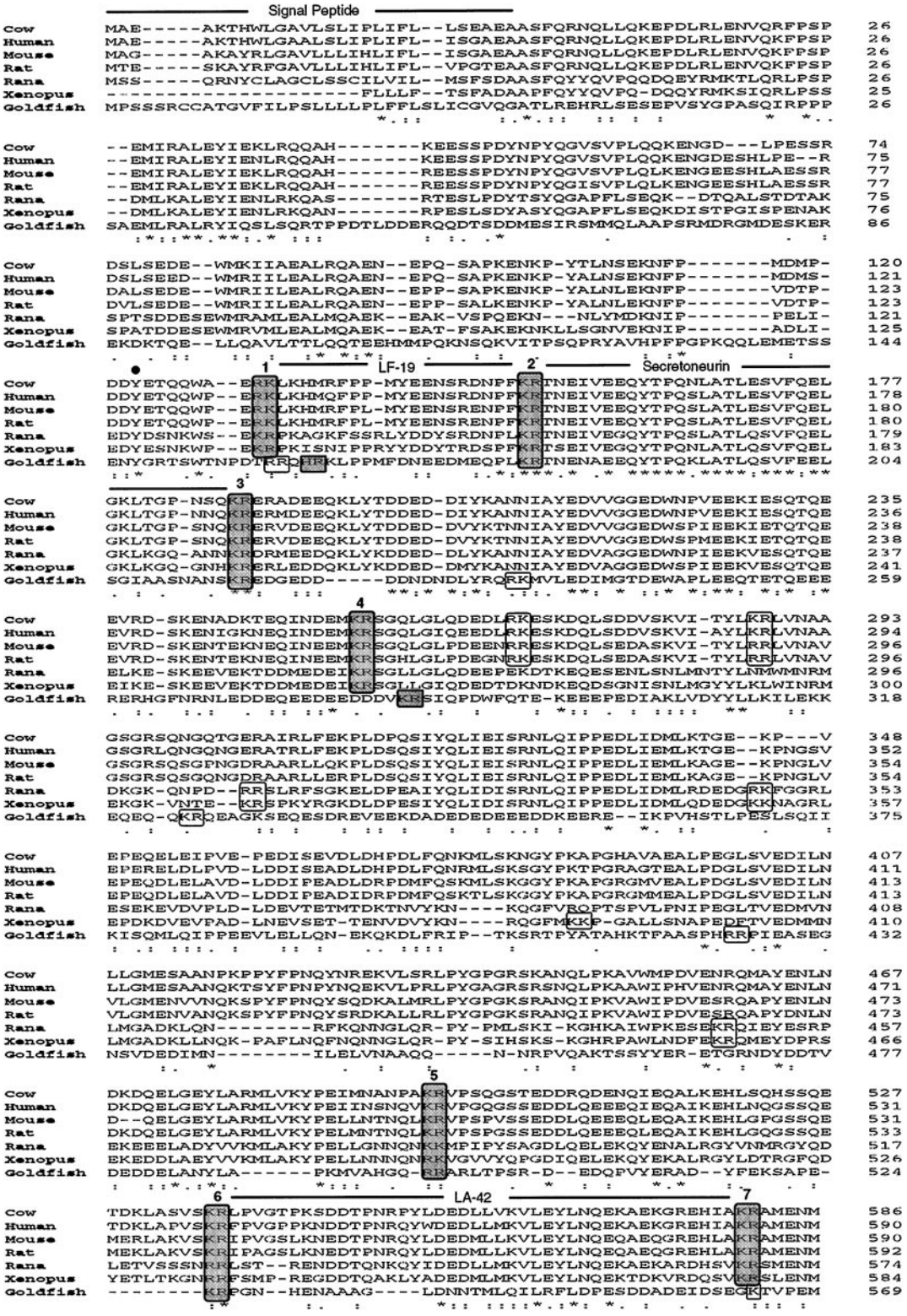

FIG. 3. Alignment of SgII amino acid sequences. The goldfish SgII sequence is aligned with deduced amino acid sequences of human, cow, rat, mouse, *Xenopus*, and *Rana* SgII. Alignment was performed using the Clustal Multiple Alignment program. *Asterisks below the alignment* represent a perfect match among the 7 sequences; *dots* represent conservative replacements (. = conserved,: = highly conserved). In addition, pairs of evolutionary conserved basic amino acids (KR, RK) appear in numbered (1–6) *shaded boxes* showing possible dibasic cleavage sites. Other potential cleavage sites appear in *open boxes*. A putative tyrosine sulfation site is marked with a *circle*. The signal peptide preceding the mature SgII molecule and three different putative bioactive peptides (LF-19, secretoneurin, and LA-42) are *overlined* along the sequence alignment. Amino acid numbering is shown at the *right* of the figure and starts at +1 following the signal peptide. GenBank accession numbers for the different SgII sequences are: cow, J05468; human, M25756; mouse, X68837; rat, M93669; rana, U68757; xenopus, X92873; goldfish, AF046002.

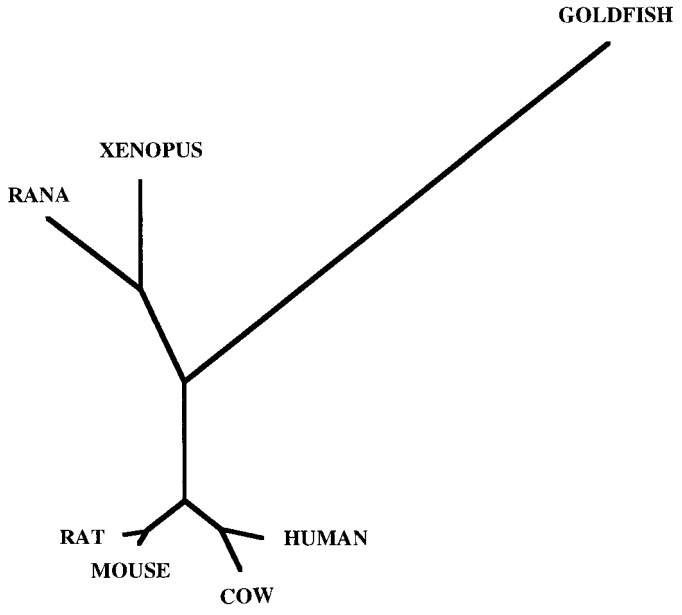

FIG. 4. Phylogenetic tree for SgII. An unrooted tree with available oligonucleotide sequences in the GenBank was drawn using the Neighbor-Joining distance method after 1000 bootstraps.

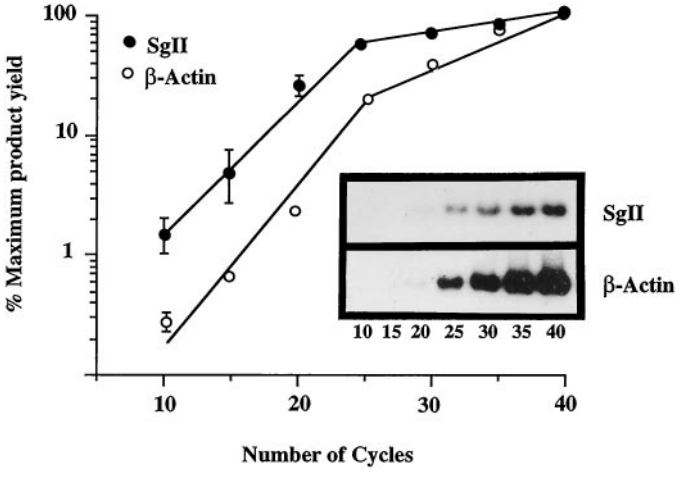

FIG. 5. Optimization of conditions to measure goldfish SgII mRNA levels by semiquantitative PCR. Total RNA (100 ng) from goldfish pituitaries was reversed transcribed to cDNA (RT-PCR) and later subjected to PCR amplifications in the presence of ^{32}P-dATP. After the indicated number of cycles, aliquots were withdrawn and subjected to acrylamide gel electrophoresis followed by autoradiography and quantified by liquid scintillation counting. Product yield was calculated as the ratio between specifically incorporated ^{32}P and total ^{32}P added to the PCR reaction. Values in the graph are expressed as percentage of the maximum product yield for each reaction (n = 6).

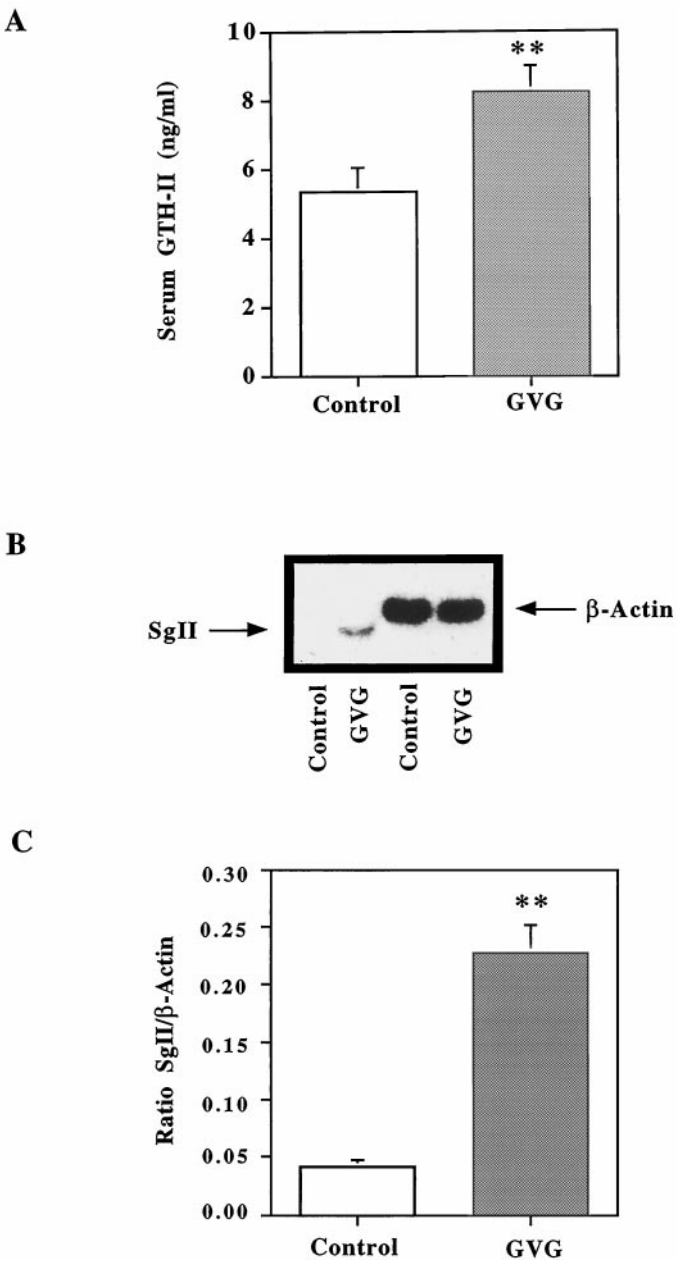

FIG. 6. Effect of GVG on serum GTH-II levels and pituitary SgII mRNA levels in goldfish. A, Sexually regressed goldfish (GSI = 1.3 ± 0.3%) in September–October) were injected ip with γ-vinyl GABA (GVG; 300 μg/g BW) or saline vehicle (control; 10 μl/g BW) and blood collected by caudal puncture 24 h later for serum GTH-II determination (n = 26 in control and n = 25 in GVG). B, Measurement of SgII expression in control and GVG treated goldfish using semiquantitative PCR. Total RNA (1 μg) was reverse transcribed to cDNA and subsequently subjected to 20 cycles of PCR amplification in the presence of ^{32}P-dATP and specific primers for SgII and β-actin. C, SgII quantitation. The ratio between SgII and β-actin was calculated using the original cpm values obtained after excising the radioactive band from the polyacrylamide gel and scintillation counting (n = 8).

Secretogranins are large acidic proteins in the secretory vesicle that undergo selective processing during regulated secretion (15, 31). Although SgII was originally found in the anterior pituitary (32), different techniques have shown that it is widely distributed throughout the mammalian endocrine and nervous system (15). As shown in the Northern blot analysis, two different transcripts of approximately 2650 bp and 2975 bp, respectively, were found in goldfish pituitary and brain. This is in contrast to other species where only a single transcript has been observed. Additionally, mRNA size is variable, ranging from 2.5 kb in human (33), which is similar to the shorter goldfish SgII mRNA, and 4.4 kb in *Xenopus* (34), which is significantly longer than both goldfish transcripts. Explanations for the appearance of two SgII transcripts in fish are speculative but could relate to differences

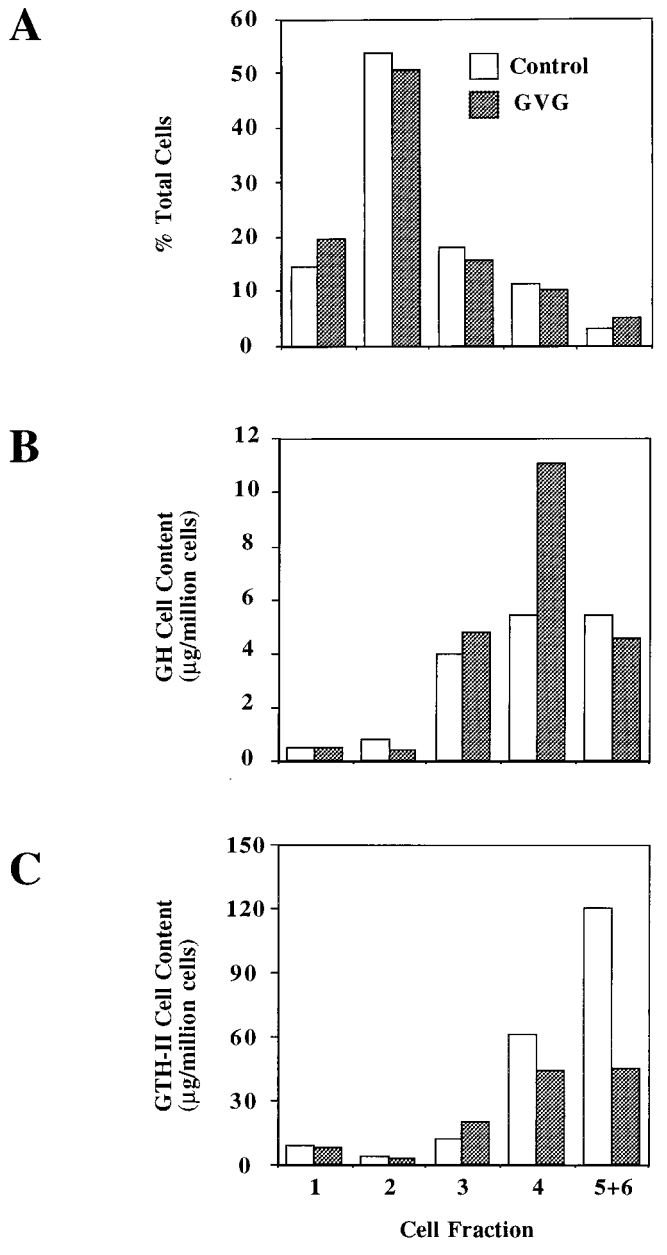

FIG. 7. Percoll gradient centrifugation of goldfish pituitary cell dispersions. A, Percentage of total number of cells in the different pituitary cell fractions after cell dispersion and Percoll gradient centrifugation. B, Cellular GH content in the different pituitary fractions. C, Cellular GTH-II content in the different pituitary fractions. Values are presented as the mean of two independent experiments.

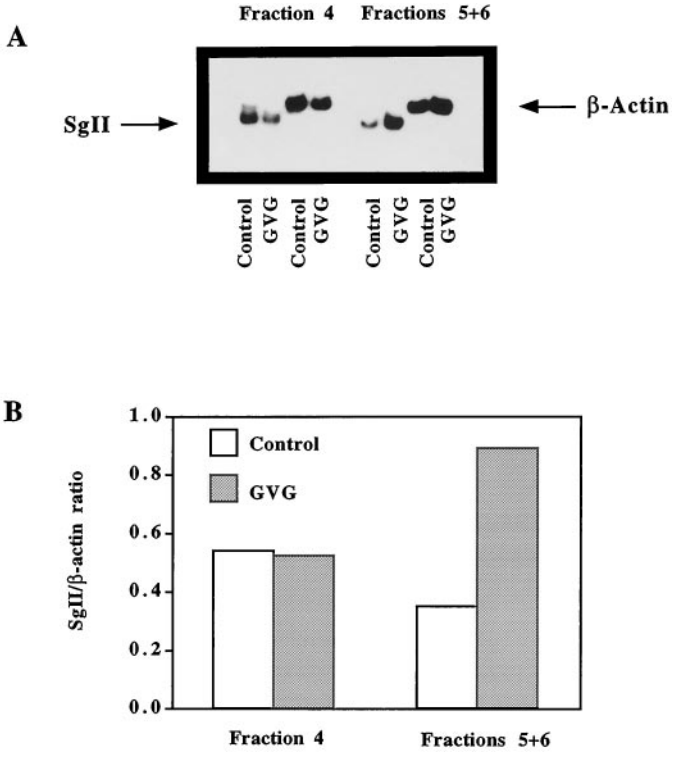

FIG. 8. Effects of GVG on goldfish SgII mRNA levels in the gonadotrophs and the somatotrophs. A, Measurement of SgII expression in the somatotroph-enriched cell fraction (fraction 4) and the gonadotroph-enriched cell fractions (fractions 5 + 6) in control and GVG treated spermiating male goldfish (July) using semiquantitative PCR. Poly(A)$^+$ RNA was reverse transcribed to cDNA in two independent RT reactions and subsequently subjected to 20 cycles of PCR amplification in the presence of ^{32}P-dATP and specific primers for SgII and β-actin. B, SgII quantitation. The ratio between SgII and β-actin was calculated using the original cpm values obtained after excising the radioactive band from the polyacrylamide gel and scintillation counting. Ratios are calculated as an average from eight different gels (four for each of two cDNA pools).

in the lengths of 5′ and 3′ untranslated regions, alternative splicing events or reflect the tetraploid genome in goldfish. Nevertheless, both SgII mRNAs were up-regulated by GVG injection.

One processed peptide derived from SgII is SN, a neuropeptide found throughout the central nervous system (22, 35, 36). This neuropeptide has been shown to have important biological activity in the mammalian central nervous system, inducing dopamine release (27, 37) and also in the immune system, having chemotatic activity for monocytes and thus contributing to neurogenic inflamatory events (38). SN is the only segment of fish SgII showing high homologies with other species. In all species, the dibasic proteolytic cleavage sites are highly conserved at the N- and C-terminals of this bioactive neuropeptide. Significantly, goldfish SN is 34 amino acids long, which contrasts with the situation in mammals and amphibians where SN is a 33 amino acid peptide. Overall, the SN peptide shows 76% similarity between fish, amphibians, and mammals. Goldfish SN is 59% identical to mammalian SN and 62% identical to *Rana* SN. Two clear groups branching independently from the SgII phylogenetic tree can be seen, one for the mammalian species and another one for the amphibians. However, goldfish SgII appears to be very different from both groups, differences that are apparent at both the nucleotide and the amino acid sequence levels.

Associated with *in vivo* GVG-induced GTH-II release was a dramatic increase in pituitary expression of SgII as determined originally in the RAP-PCR and independently confirmed by Northern blot and semiquantitative PCR. It was important to determine the likely cell type responding to activation of the hypophysiotropic GABA system. For this purpose, we used pituitary cell dispersion and Percoll gradient centrifugation to obtain semipurified endocrine cell

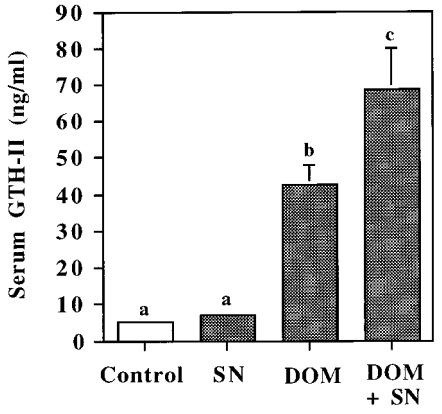

FIG. 9. Effects of SN and domperidone on GTH-II release *in vivo* in the goldfish. Sexually regressed fish were ip injected with the DA receptor antagonist domperidone (10 μg/g BW) in DMSO vehicle (1 μl/g BW) 24 h before experimentation. Controls received an equivalent volume of DMSO. The following day, fish were injected ip with synthetic goldfish SN (1 μg/g BW) or 0.6% saline vehicle (1 μl/g BW; pH = 6.4). Blood samples were collected 30 min after SN injection and plasma GTH-II levels measured by RIA. Values are expressed as mean + SEM (n = 22–23). *Different letters* represent statistical differences ($P < 0.05$). The results are representative of the same experiment performed on two separate occasions.

fractions. Consistent with the stimulatory effect of GVG on *in vivo* GTH-II release was a 60% decrease in cellular GTH-II content in the gonadotroph-enriched fraction and a 250% increase in SgII expression the same fraction. Thus, increased serum GTH-II levels, depletion of cell GTH-II content, and concomitant increase in SgII expression are consistent with the idea that GABA enhances GTH-II secretion in adult goldfish. This is also in line with other studies in the goldfish showing that type A and type B GABA receptor agonists increase serum GTH-II levels *in vivo* (8). Our data also show a significant increase in GH content in the somatotroph enriched cell fraction following GVG injection, probably due to an inhibition of GH release by GABA in goldfish (39). In fish, in contrast to mammals, there is significant cellular regionalization within the pituitary. Gonadotrophs and somatotrophs are colocalized in the pars distalis of the goldfish pituitary, and both are innervated by GABA neurons (6). The up-regulation of SgII mRNA levels in the gonadotrophs is in contrast to any apparent effect on SgII in the somatotrophs, indicating specificity of the SgII response to GVG.

Previous work has demonstrated that rat gonadotrophs co-release SgII-immunoreactive substances (including SN) and LH upon stimulation by the hypothalamic neuropeptide GnRH (14). Although we have not yet measured release of SgII-related peptides, we hypothesize that SN may be secreted from the stimulated goldfish gonadotroph, as it occurs in the rat. This lead us to test the possible *in vivo* bioactivity of synthetic goldfish SN. Injection of SN did not affect serum GTH-II in sexually regressed goldfish when given alone. However, this was not surprising because GTH-II release is under a potent tonic inhibition by dopamine in goldfish (9). Thus, animals were pretreated with the specific DA_2 receptor antagonist domperidone to inhibit DA action in the gonadotrophs, and in these animals, SN induced a robust increase of GTH-II serum levels. The magnitude and in particular the rapidity of the effect of the new form of SN indicates that this is a biologically significant response. It has previously been shown that two processed peptides derived from another chromogranin, chromogranin A, are regulators of the endocrine cell function (40). Pancreastatin, a 49 amino acid peptide inhibits insulin release from the pancreas (41) and a second derived peptide chromostatin, a 20 amino acid peptide, inhibits catecholamine secretion from chromaffin cells (42). Although experimental evidence is still lacking, it has also been proposed that SgII may be involved in packaging of LH into the secretory vesicles (15). We now suggest that SN could have important stimulatory effects on GTH-II release *in vivo*, linking the regulated secretory pathway with positive feedback control of gonadotroph function. The mechanism by which SN stimulates GTH-II release was not addressed in the present study. No SN receptors have yet been identified in any species, and proposed mechanisms of action are at best speculative. Nevertheless, it does not appear to involve actions on DA nerve terminals in the goldfish pituitary because domperidone did not block SN action. Alternatively, SN could stimulate GnRH release or have some direct effects on gonadotrophs in the goldfish pituitary.

We have also isolated several other mRNA transcripts stimulated by GABA. A goldfish homolog of a human GTPase activating protein reported to be involved in several processes, especially exocytosis was identified. Significantly, we have also obtained the sequence of the goldfish homolog of FKBP-12 rapamycin-associated protein (FRAP), which shares 79% identity with human FRAP. Originally characterized as a protein involved in the effects of the immunosuppressant rapamycin, a well known inhibitor of p70-(S6)-kinase (43) and G_1 progression of the cell cycle (44), FRAP is integral to many signal transduction pathways (45), appears most often associated with phosphatidylinositol-3-kinase and Ca^{2+} (46), and has been shown to contain a putative phosphatidylinositol kinase domain (44). A potential homolog of mouse p85α subunit of the phosphatidylinositol-3-kinase complex was also identified. Goldfish p85α subunit was highly expressed in the pituitary of controls and was not affected by GVG injection. This transcript presented the lowest homology to accessible GenBank sequences of all the fragments obtained with the RAP-PCR. This fragment showed only 28% identity (63% similar to its mouse counterpart) in the region of p85α located between the p110 binding site and the SH2 domain (47). Additionally, goldfish homologs of an arginine-rich nuclear protein and a polypirimidine tract binding protein were also up-regulated by GVG. These molecules are involved in mRNA processing, alternate splicing (48), and translation (49) and their increased expression associated with GVG-stimulated GTH-II release and enhanced activity of secretory pathways is likely to play an important role in the synthesis of many other proteins in the goldfish pituitary.

In conclusion, we demonstrate for the first time that the so-called inhibitory neurotransmitter GABA stimulates a novel SgII in fish. Activation of endogenous hypophysiotropic GABA systems induce a specific increase of SgII mRNA expression in pituitary gonadotrophs, concomitant with a

decrease in GTH-II cell content and increased GTH-II release *in vivo*. This suggests that SgII could be acting as a molecular chaperone for hormone packaging in the regulated secretory vesicle. Sequence comparisons with the known SgII molecules indicate that the tyrosine sulfation motif and only 6 out of 7 potential dibasic cleavage sites are conserved in fish, amphibians, and mammals. These data indicate that processed peptides derived from goldfish SgII will likely be secreted. The highest homologies are found within the SN region, suggesting that this neuropeptide is of likely biological importance in the vertebrates. Indeed, we demonstrate for the first time that SN stimulates reproductive function by increasing serum GTH-II levels in male and female goldfish. Activation of gonadotrophic cell function by GABA or other neurohormones may lead to secretion of SN, which in turn promotes further release of GTH-II by an as yet uncharacterized secretion-coupled autocrine mechanism. Furthermore, although many aspects of SgII function and up-regulation of other secretion-related proteins remain to be fully understood, it is possible that SN has additional paracrine roles to modulate other cell types within the pituitary complex.

Acknowledgments

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