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Role of Basolateral Amygdalar Somatostatin 2 Receptors in a Rat Model of Chronic Anxiety

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Abstract

Repeated exposure to stress has been implicated in inducing chronic anxiety states. Stress related increases in anxiety responses are likely mediated by activation of corticotropin-releasing factor receptors (CRFR) in the amygdala, particularly the basolateral amygdala (BLA). Within the BLA, acute injections of the CRFR agonist urocortin 1 (Ucn1) leads to acute anxiety, whereas repeated daily injections of subthreshold-doses of Ucn1 produces a long-lasting, persistent anxiety-like phenotype, a phenomenon referred to as Ucn1-priming. Relative gene expressions from the BLA of vehicle and Ucn1-primed rats were analyzed with quantitative RT-PCR using a predesigned panel of 82 neuroscience-related genes. Compared to vehicle-primed rats, only expression of the somatostatin receptor 2 gene (Sstr2) was significantly reduced in the BLA of Ucn1-primed rats. The contribution of Sstr2 on an anxiety phenotype was tested by injecting a Sstr2 antagonist into the BLA in un-primed rats. The Sstr2 antagonist increased anxiety-like behavior. Notably, pretreatment with Sstr2 agonist injected into the BLA blocked anxiety-inducing effects of acute Ucn1 BLA-injections and delayed anxiety expression during Ucn1-priming. However, concomitant Sstr2 agonist pretreatment during Ucn-1 priming did not prevent either the development of a chronic anxiety state or a reduction of BLA Sstr2 expression induced by priming. The data demonstrate that the persistent anxiety-like phenotype observed with Ucn1-priming in the BLA is associated with a selective reduction of Sstr2 gene expression. Although

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Sstr2 activation in the BLA blocks acute anxiogenic effects of stress and down-regulation of BLA Sstr2, it does not suppress the long-term consequences of prolonged exposure to stress-related challenges.

Keywords

Neuropeptide; social interaction; anxiety; amygdala

INTRODUCTION

Repeated or prolonged exposure to stress can lead to anxiety disorders in humans (Arborelius et al. 1999; Haller 2001) and increased anxiety-like responses in rodents (Doremus-Fitzwater et al. 2009; Shoji and Mizoguchi 2010; Vyas and Chattarji 2004). Stress-induced increases in anxiety-like behavior have been associated with neuronal plasticity in limbic structures including the amygdala (McEwen et al. 2012; Vyas et al. 2002), which plays a critical role in mediating anxiety responses (Amat et al. 2016; Phelps and LeDoux 2005; Rauch et al. 2003; Shekhar et al. 2005). Anxiety-like responses, both behavioral and physiological, are associated with amygdalar activation (Amat et al. 2016; Anand and Shekhar 2003; Felix-Ortiz and Tye 2014; Rainnie et al. 2004; Rauch et al. 2003; Rosenkranz et al. 2010; Sajdyk and Shekhar 1997; Sanders and Shekhar 1995). Within the amygdala, the basolateral amygdala (BLA) is particularly important for chronic anxiety-like responses and a putative site of action for anxiolytic drugs, such as selective serotonin reuptake inhibitors (Abrams et al. 2005; Sim et al. 2010) and benzodiazepines (Johnson et al. 2010; Sanders and Shekhar 1995; Scheel-Kruger and Petersen 1982).

Corticotropin-releasing factor (CRF), a 41 amino acid neuropeptide, is associated with stress and increasing both BLA neural activity and anxiety-like behavior (Sajdyk et al. 1999; Walker and Davis 1997). CRF release into the amygdala is increased by stress (Cook 2004; Koob and Heinrichs 1999) and stress-induced behavioral changes are likely due to activation of CRF 1 receptors (CRFR1) (Gehlert et al. 2005; Heinrichs et al. 1997; Timpl et al. 1998). Furthermore, increases in anxiety-like behavior, resulting from exposure to stress, have been postulated to be a consequence of amygdala CRFR activation (Davis et al. 2010; Lee and Davis 1997; Rainnie et al. 2004; Sajdyk and Gehlert 2000; Sajdyk et al. 1999; Sterrenburg et al. 2011). Acute bilateral microinjection of the CRFR agonist, urocortin 1 (Ucn1) into the BLA of rats, induces acute anxiety-like behavior in multiple behavioral tests (Rainnie et al. 2004; Sajdyk et al. 1999) and this anxiogenic response is blocked with CRFR1 antagonist pretreatment into the BLA (Gehlert et al. 2005).

In order to model the mechanisms by which repeated stress episodes, and resultant CRF release within the amygdala can induce persistent anxiety-phenotypes, Sajdyk and co-workers (1999) gave repeated Ucn1 (6 fmole/100 nl/side, a dose that is acutely sub-anxiogenic) injections bilaterally into the BLA for five consecutive days. This treatment paradigm, referred to as Ucn1-priming, results in: 1) behavioral sensitization, exhibited by a significant anxiety response to the subthreshold dose of Ucn1 after the third priming injection; 2) persistent anxiety-like phenotype which lasts several months after the fifth

Ucn1 injection; 3) a significant reduction in tonic inhibition of BLA-projection neurons and an increase in BLA network excitability; and 4) panic-like responses to subthreshold doses of sodium lactate (Gehlert et al. 2005; Rainnie et al. 2004; Sajdyk and Gehlert 2000; Truitt et al. 2007) and other treatments related to panic disorders in humans (reviewed in Johnson et al. 2014; Truitt et al. 2007). Collectively, these data suggest that Ucn1-priming in the BLA induces long-term plasticity within the amygdala resulting in a persistent anxiety-like and panic-prone phenotype. However, the mechanisms by which Ucn1-priming induces these stress-like behavioral effects within the BLA has yet to be determined, and thus the model lacks construct validity as a stress-like model.

Another key neuropeptide system that regulates anxiety within the BLA is somatostatin, acting principally through the Somatostatin receptor 2 (Sstr2) (Gehlert et al. 2005), which is a G-protein coupled receptor that is negatively coupled to adenylyl cyclase. Exposing a rat to a predator (ferret) down-regulates Sstr2 mRNA in the amygdala (Nanda et al. 2008). Perhaps the Ucn-1 priming model of chronic anxiety works through mechanisms similar to this ethologically relevant model of chronic anxiety. Somatostatin (Sst) is a neuropeptide with five receptor subtypes that only recently has been implicated in the rodent stress response (Stengel et al. 2013). Sstr2 knock out mice have increased anxiety (Viollet et al. 2000) and selective Sstr2 antagonism in the BLA blocks Sst-induced anxiolytic-like effects (Yeung and Treit 2012). Chronic mild stress of rats reduced Sstr2 binding in the BLA to a greater degree than several other brain regions (Faron-Gorecka et al. 2016). As Sstr2 has functional interactions with other Sst receptors (Aourz et al. 2011), we investigated the impact of Ucn-1 priming on all Sst receptors and several other neuroscience-related genes of interested on a predesigned panel (Table 1). Several other genes were examined for numerous scientific justifications, such as neuropeptide Y receptors suspected for an anxiolytic role on Sst BLA interneurons (Mackay et al. 2019).

Here, we report our initial studies on the mechanisms by which Ucn1-priming of the BLA increases anxiety-related behaviors. Among the 84 genes measured, we identified a selective and persistent reduction in Sstr2 mRNA levels in the BLA following Ucn1-priming and demonstrate Sstr2 antagonism increases anxiety. Next, Sstr2 agonist was found to override acute Ucn1-induced anxiety as well as delay behavioral sensitization of the anxiety phenotype induced by Ucn1-priming. Finally, we observed that blocking the experience of repeated anxiety via Sstr2 agonists during Ucn1-priming failed to prevent the downregulation of Sstr2 mRNA or the persistent anxiety-like phenotype induced by Ucn1-priming. These experiments suggest Ucn1-priming induces persistent anxiety-like state via a mechanism similar to that produced by chronic stress and a novel role of Sstr2 modulating a BLA circuit in a model of chronic anxiety, increasing the construct validity of this model.

EXPERIMENTAL PROCEDURES

General Methods

Animals.—All experiments used male Wistar rats between 275-300g (Harlan Laboratories, Indianapolis, IN). Rats were individually housed, provided food and water *ad libitum*, and maintained at standard environmental conditions (22°C; 12-12 hour light/dark cycle; lights on at 7:00 A.M.). All experiments were conducted in accordance with the Guide for the

Care and Use of Laboratory Animals (National Research Council (U.S.). Committee for the Update of the Guide for the Care and Use of Laboratory Animals. et al. 2011).

Surgery.—Five to ten days after arrival, rats were anesthetized with isoflurane (MGX Research Machine; Vetamic, Rossville, IN, USA), then a 26-gage microinjection guide cannula (Plastics One, Roanoke, VA) was sterotaxically implanted bilaterally towards the BLA [incisor bars: −3.3 mm; AP: −2.1; ML ±5.0 relative to bregma and DV −8.0 (Paxinos and Watson 2005)]. All rats were given at least four days recovery.

Intracranial microinjections.—All compounds were administered intracranially (i.c.) into the BLA: CRF1/2R agonist, Ucn1; Sstr2 antagonist, CYN-154806 [Ac-4NO2-Phe-c(DCys-Tyr-DTrp-Lys-Thr-Cys)-D/LTyr-NH2]; [(Tocris Cookson, Inc; Ellisville, MO); nicotinic receptor subtype (α4β2nACh) antagonist, Dihydro-beta-erythroidine hydrobromide (DHβE) (Sigma; St. Louis, MO); Sstr2 agonist, BIM-23027 (American Peptide Company, Inc; Sunnyvale, CA)]. All compounds were dissolved in a 1% Bovine Serum Albumin (BSA: Sigma; St. Louis, MO; Veh) and delivered bilaterally into the BLA through 33 gauge microinjector that extended 1mm below the guides (Plastics One, Roanoke, VA). Drugs were microinfused at a volume of 100nl delivered over 30s via a syringe pump (Havard Apparatus, Holliston, MA, Model PHD 2000). The injectors remained in place for one additional minute (Sajdyk et al. 1999). After completion of each study, rats were sacrificed and injection sites were verified as previously described (Sajdyk et al. 1999) or (Johnson et al. 2010) for rats used in RT-PCR experiments.

Priming protocol.—Priming protocols were administered as previously described (Rainnie et al. 2004; Shekhar et al. 2003; Truitt et al. 2007). Forty-eight hours after baseline social interaction (SI) testing, priming injections were administered bilaterally into the BLA once per day for five consecutive days, between 7:30 and 10:30 am.

Social interaction (SI) test.—The SI test is a fully validated test of experimental anxiety-like behavior in rats (Sanders and Shekhar 1995; Shekhar and Katner 1995) that is sensitive to FDA approved treatments for anxiety disorder symptom management that include benzodiazepines (Johnson et al. 2010) and selective serotonin reuptake inhibitors (Lightowler et al. 1994). The SI test was performed as previously described (Lungwitz et al. 2014). Briefly, rats are acclimated to the environment and handling for several days prior to any SI testing. Each SI session consisted of a 5 min exposure to a novel, age, weight and sex match conspecific. During the SI test, the “experimental” rat and an unfamiliar “partner” rats were placed together in the center of the box, and the total duration (sec) of non-aggressive physical contact (grooming, sniffing, crawling over and under, etc.) initiated by the “experimental” rat was quantified over a 5 min duration. SI testing was performed 2 or 3 days (noted for each experiment below) after collection of a baseline (baseline is usually collected on Friday and SI test 1-5 collected the following Mon-Fri) between 8:00 a.m. and 1:00 p.m., under low light conditions (40 watt red light) and in a familiar SI testing arena (91.5cm L x 91.5cm W x 30.5cm H).

Experimental Methods

Experiment 1: Ucn1-priming, behavior and gene expression in the BLA.—Three days after a baseline SI test was administered, priming injections were initiated; rats received either 5 daily bilateral BLA-injections of vehicle (100nl distilled water containing 1% Bovine Serum Albumin (BSA)/side) or Ucn1 (6 fmol in 100 nl distilled water containing 1% BSA/side). Three days after the last (5^{th}) priming injection, anxiety-like behavior was once again assessed in the SI test (Post-priming session, Day 8). Two days (Day 10) following the post-priming SI session rats were sacrificed and brains processed for quantitative real-time polymerase chain reaction (qRT-PCR) analysis.

Tissue collection and processing.—Determination of changes in gene expression within the BLA was examined in the above rats as an attempt to identify putative cellular mechanisms of the lasting increased anxiety observed with Ucn1-priming. Rats were sacrificed two days after the post-priming SI test (10 days after the first priming injection) and BLA tissue processed for RT-PCR analysis. The relative gene expression from the BLA of Veh- and Ucn1-primed rats was analyzed with qRT-PCR using a predesigned panel of 82 neuroscience genes (see Table 1). The bilateral BLA samples were dissected from two adjacent 300μm thick coronal frozen sections [−2.0 and −2.8 relative to bregma (Paxinos and Watson 2005)] using 0.96mm diameter tissue punch (Vibratome). RNA isolation and reverse transcription into cDNA was performed as previously described (Johnson et al. 2010). Briefly, RNA was isolated from the collected BLA tissue samples with RNeasy Micro Kits (QIAGEN, Valencia, CA). Starting RNA concentrations were adjusted to 100 ng/20μl and RNA was reverse transcribed into cDNA using Reaction Ready™ First Strand cDNA Synthesis Kit (QIAGEN). The cDNA was diluted to a final volume of 1 ng/μl with RNase-free water. Efficiency was confirmed by amplification of the endogenous control gene beta-Actin with the GeneAmp RNA PCR Core Kit (Applied Biosystems, Foster City, CA).

Neurotransmitter Receptors and Regulators RT^{2}Profiler™ PCR Array analysis.—The mRNA expression from the BLA of veh- and Ucn1-primed rats was determined with relative qRT-PCR using a predesigned panel of 84 neuroscience genes [Neurotransmitter Receptors and Regulators RT^2*Profiler*™ PCR Array (QIAGEN)]. This array consists of a 96 well plate with each well containing the primers for a single neuroscience or control gene (Table 1).

The cDNA was added to RT^2 Real-Time™ SYBR Green/ROX PCR Master Mix [1ng cDNA/25μl master mix (Quigen)] and 25μl of this solution was added to each well of Neurotransmitter Receptors and Regulators RT^2*Profiler*™ PCR Array. The cycling conditions were 50 °C for 2 minutes, 95 °C for 10 minutes then 40 cycles of 95 °C for 15 seconds, 65 °C for 15 seconds, and 72 °C for 1 minute in an Eppendorf Mastercycler® ep *realplex* instrument (Eppendorf, Westbury, NY).

qRT-PCR expression.—The average Ct of four reference genes [ribosomal protein, large, P1 (Rplp1), ribosomal protein L13A (Rpl13a), beta-Actin (Actb) and lactate dehydrogenase A (Ldha)] was used for normalization and the Veh primed group served as the relative

control group. Relative quantification (RQ) of each gene was determined using $RQ = 2^{-\Delta\Delta Ct}$ with the aid of the custom software provided with the Neurotransmitter Receptors and Regulators RT^2*Profiler*™ PCR Array.

Experiment 2: Sstr2 antagonist in the BLA and Anxiety-like behavior.—Two days following baseline and prior acclimation sessions, rats received intra BLA infusions of either vehicle (1% BSA, 100nl) or the selective Sstr2 antagonist, CYN-154806 [(CYN) 1 or 10 pmol in 100 nl/side] 30 mins prior to their first SI test. These doses were selected based on a review of the literature (Feniuk et al. 2000).

Experiment 3: Sstr2 agonist infusion into BLA and acute Ucn1-induced anxiety-like behavior.—All rats underwent acclimation and a baseline SI test sessions. Two days later rats received bilateral intra-BLA infusions of either vehicle (1% BSA 100nl/side; Veh) or the Sstr2 agonist, BIM-23027 [90 pmoles/100 nl/side; BIM(90)] followed 30 min later by bilateral intra-BLA infusion of Ucn1 [100 fmoles/100 nl/side; Ucn1(100)] or Veh, (1%BSA 100nl/side). Thus, the following combination of infusions were administered: Veh-Ucn1(100), BIM(90)-Ucn1(100) and BIM(90)-Veh. Thirty mins after the second infusion rats underwent SI testing with a novel conspecific as described above. The effect of BIM-23027 without Ucn1 treatment was investigated in a separate group of rats. Rats received a baseline SI test, which was no different than the previous baseline for experiment 3 ($p>0.05$), and then were treated with BIM(90) 30 min prior to a vehicle injection as described above.

Experiment 4: Sstr2 agonist and Ucn1-priming.—This study followed the same procedure described in experiment 1 except that 30 min prior to each of the five priming infusions (5x) rats received bilateral intra-BLA infusion of vehicle (1% BSA 100 nl/side; Veh) or BIM [90 pmoles/100 nl/side; BIM(90)]. Each of the 5 priming infusions consisted of either 1% BSA (Control, 5x) or 6 fmol of Ucn1 [Ucn1(6), 5x)]. This resulted in three priming conditions Veh-Control, 5x; Veh-Ucn, 5x and BIM(90)-Ucn1(6). Here, SI time was measured 3 days prior to priming (Baseline), and 30 min following the 1st, 3rd and 5th priming infusion (Infusion day 1, day 3 and day 5) as well as 3 days after the last priming infusion (Post-priming, day 8).

To determine the effect of BIM-23207 pretreatment during Ucn1-priming on the Sstr2 mRNA expression, the BLAs from a subset of animals used in Experiment 4 were collected five days after priming ceased (day 10) and tissue was processed for qRT-PCR, as described above. Sstr2 gene expression was determined using absolute qRT-PCR methods and normalized to beta-actin expression following previously described methods (Johnson et al. 2010). Briefly, PCR product was generated using the primers for Sstr2 or beta-Actin listed in Table 2, then cloned into a pCR®4-TOPO vector (Ctlg. # K457502, ThermoFisher Scientific). The desired gene fragments were purified, amplified and used for generation of standard curves for both the Sstr2 and beta-actin genes.

After purification, the plasmid DNA concentration was measured using a ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). The mass of a single plasmid molecule was determined by multiplying the plasmid concentration by the total

number of base pairs contained in the plasmid. The mass of plasmid DNA needed for the specific PCR product was calculated [300,000 copies for Sstr2, 3,000,000 for beta-Actin] and serial dilutions for the standard curve were prepared accordingly. The C_t value of each sample was used to calculate the log input amount based on the slope and y-intercept of the standard curve line. The copy number for each sample $=10^{[\log \text{input amount}]}$. The ratio of Sstr2 to beta actin for each sample was used in the data analysis to control for total tissue content.

Data analysis

Behavior.—SI times for the priming studies (experiments 1 and 4) were analyzed using a Two-way repeated measure ANOVA, with main factors of day and treatment condition. Post hoc comparisons within treatment conditions were made with Dunnett's when comparing SI time back to baseline SI time. Between group analysis was determined using Bonferroni multiple comparisons or Fisher's LSD post hoc tests where appropriate. For experiment 2 a one-way ANOVA was used for comparison between drug treatments. repeated measures ANOVA or a paired ttest were used in experiment 3 where appropriate. Where needed, these data were corrected for sphericity using the Geisser-Greenhouse method. For analyses with groups where $n < 5$, non-parametric statistics were used [Kruskal-Wallis (ANOVA), Dunn's (post-hoc), Wilcox for within comparisons and Mann-Whitney for between comparisons]. Where appropriate (parametric analyses), post hoc comparisons were made using Tukey's. A $p<0.05$ was the cutoff to determine significance. All statistics were conducted with Prism (GraphPad Software, Inc).

Gene-expression analysis.—For experiment 1, differential gene expression by treatment group was determined using a Two-way ANOVA with main factor of gene and priming condition. Kruskal-Wallis one way non-parametric analysis was used to compare genes of interest in vehicle primed rats vs. Ucn-1 primed rats in experiment 1 and drug treatment groups in experiment 4. Of the 82 genes screened post-priming (see Table 1), 10 had expression values below our preset threshold limit (Ct=34) and were removed from any further analysis. To protect for multiple comparisons, the criteria for inclusion in further linear comparisons were set a priori at a conservative minimum of at least ±1.5 fold change in expression relative to Veh-primed rat. For linear comparisons and more stringent analysis of the expression of the genes of interest, RQ values were converted to Log base 10, and comparisons for expression levels of these genes between Veh-primed and Ucn1-primed rats were made using a one-way non-parametric test with correction (Kruskal-Wallis). When appropriate, Dunn's post hoc test separated the effects of the pretreatment conditions on mRNA expression.

RESULTS

Experiment 1: Ucn1-priming, behavior and gene expression in the BLA.

Ucn1-priming in the BLA (see Fig. 1 for injection site location) resulted in a persistently increased anxiety-like behavior, as determined by the SI test (Fig. 2a). For experiment 1, there was a significant day effect and treatment by day interaction (repeated measures 2-way ANOVA $F_{(1,7)}=5.92$, $P= 0.03$ and $F_{(1,7)}=29.19$, $P= 0.001$; respectively). Rats primed with Ucn-1 had significantly reduced SI times (anxiety-like) 3 days after the last injection

(post-priming) compared to Veh-primed rats post-priming (Mann Whitney test p= 0.015) as well as pre-priming baseline SI times (Wilcox's p=0.031).

Determination of changes in gene expression in the BLA was examined to identify putative cellular mechanisms of the lasting increases anxiety observed with Ucn1-priming. A main effect of treatment on overall gene expression was observed (two-way ANOVA $F_{1,71}$=7.263, p=0.005) where Ucn1-primed rats had an overall reduction in gene expression compared to Veh-primed rats. To protect for multiple comparisons, these criteria were set at a conservative minimum of at least ±1.5 fold change in expression relative to Veh-primed rat and a greater than 95% confidence interval ($p < 0.01$, Fig. 2B). Four genes met these criteria, somatostatin receptors 2 (Sstr2) and 4 (Sstr4), cholinergic nicotinic receptor subunit alpha4 (Chrna4) and cholinergic muscarinic receptor 4 (Chrm4). Relative to Veh-primed rats only the Sstr2 expression was significantly reduced following Ucn-1 priming (Kruskal-Wallis, p = 0.002, Dunn's multiple comparisons post Hoc, p = 0.029, Fig. 2C).

Experiment 2: Sstr2 receptor antagonist in the BLA and anxiety-like behavior.

The next goal was to determine if acutely antagonizing the Sstr2 receptors in the BLA increased anxiety-like behavior. Intra-BLA injections of the selective Sstr2 antagonist, CYN-154806, significantly reduced SI times (increase anxiety) compared to vehicle (n=7) injected rats at the 1 (n=5) and 10 (n=3) pmol doses (ANOVA $F_{2,14}$=6.191, P=0.0142; Tukey's p=0.026 and p=0.044 for 1 pmol and 10 pmol respectively; Fig. 3).

Experiment 3: Sstr2 agonist infusion into BLA and acute Ucn1-induced anxiety-like behavior.

The possibility of increasing BLA Sstr2 activity to override an acute BLA injection of an anxiogenic Ucn1 dose was investigated. Bilateral intra-BLA injections of 100 fmol Ucn1 significantly reduced SI times in rats receiving intra-BLA pre-injections of vehicle [Veh-Ucn1(100), n=5)], compared to baseline (repeated measures ANOVA $F_{2,8}$=5.941, P=0.041; Dunnett's q = 2.914 p = 0.035; Fig 4). This reduction in SI time, however, was blocked in rats pretreated with the 90 pmol dose, of the Sstr2 agonist, BIM-23027, 30 minutes prior to Ucn1 [BIM(90)-Ucn1(100), n=5; Tukey's q=3.833, p=0.044]. The affect of BIM-23027 without Ucn1 treatment was investigated in a separate group of rats (n=5). Rats received a baseline SI test and then were treated with BIM(90) 30 min prior to a vehicle injection as described above. Here, BIM(90) did not alter SI time from its own baseline (data not shown; paired ttest, t=0.992, p=0.377).

Experiment 4: Sstr2 agonist and Ucn1-priming.

Next, the effect of Sstr2 agonist pretreatment in the BLA was investigated in conjunction with Ucn1-priming. There were three treatment groups as described above Veh-Ucn1(6), (n=6), BIM(90)-Ucn1(6) (n=6), and Veh-Control (n=7). There was a significant Treatment, Day, and Treatment by Day interaction (2-Way repeated measures ANOVA $F_{2,16}$=3.949, P=0.040, $F_{4,64}$=4.95, P=0.001 and $F_{8,64}$=2.110 P=0.047, respectively; Fig. 5a). A sensitized anxiety-like response was observed in the Veh-Ucn1(6) group, defined as significant reduction in SI time from baseline and from Veh-control rats on priming days 3 and 5 (Dunnett's $q \geq 4.05$, $p \leq 0.002$; Tukey's $q \geq 3.808$ $p \leq 0.023$, respectively). Also for

Veh-Ucn1(6) group, a persistent anxiety-like phenotype was observed as SI times were still significantly reduced 72 hours after last injection compared to baseline and Veh-control rats (Dunnett's $q \geq 3.21$ $p \leq 0.008$; Tukey's $q = 3.88$, $p = 0.020$). Administration of the Sstr2 agonist prior to Ucn1 priming [BIM(90)-Ucn1] blocked the typical reduction in SI time by priming day 3, but by post priming day 8 significantly reduced SI times compared to baseline and Veh-Control rats 72 hours after last injection were observed (Dunnett's $q=3.206$, $p=0.0077$ and Tukey's $q=3.671$, $p=0.030$). These results suggest that Sstr2 agonist administration delayed the acquisition of an anxiety-like response to Ucn1-priming, but the anxiety-like phenotype appeared 72 hours later on post-priming day 8.

BLA tissue from a subset of animals (n=3/group) in this experiment was collected five days after priming ceased (Day 10) and processed for absolute qRT-PCR. Expression of Sstr2 was significantly reduced in both Veh-Ucn1(6) and BIM(90)-Ucn1(6) groups compared to Veh-control group [one-way ANOVA Kruskal-Wallis test statistic = 5.60, $p=0.050$; Dunn's $p=0.034$ (Veh-Ucn1(6) and $p=0.043$ (Bim-Ucn1); Fig. 5b], suggesting further that Sstr2 agonist treatment only briefly masked the development of an anxiety-like phenotype.

DISCUSSION

This study identified a novel molecular mechanism within the BLA that is involved in chronic anxiety-like effects following Ucn1-priming. Consistent with previous reports, Ucn1-priming induced a persistent anxiety-like phenotype in rats (Johnson et al. 2013; Rainnie et al. 2004; Sajdyk and Gehlert 2000; Sajdyk et al. 1999; Truitt et al. 2007). Two days after the last behavioral test, BLA tissue from these rats were screened for expression of 84 neurotransmitter and neuropeptides related genes. Based on our a priori definitions for gene changes and post-hoc comparisons between treatment groups, of the 84 genes screened, only the Sstr2 gene was identified as putative candidate for a molecular mechanism of Ucn1-priming-induced persistent anxiety-like phenotype. Interestingly, the reduced expression of Sstr2 in the BLA of Ucn1-primed rats observed here is similar to previous results where chronic stress also reduced Sstr2 gene expression in the BLA of rats (Faron-Gorecka et al. 2016) suggesting Ucn1-priming and chronic stress exposure share a common mechanism. This reduction in Sstr2 gene expression in the BLA following Ucn1-priming may be due to a whole organismic response, considering Ucn1-priming exposure results in changes to the rat's behavior. However, local molecular changes to the BLA induced by Ucn1-priming may reduce Sstr2 expression and this in turn could be driving the behavioral changes. Indeed, selectively inhibiting Sstr2 acutely in the BLA with the Sstr2 antagonist, CYN-154806, increased anxiety-like behavior. This is consistent with previous reports of increased anxiety-like behavior in Sstr2 knock out mice (Viollet et al. 2000) and Sstr2 antagonism attenuates the anxiolytic effects of Sst i.c.v. infusions in the BLA (Yeung and Treit 2012).

In addition to inducing a persistent anxiety-like phenotype, Ucn1-priming reduces inhibitory potentials recorded from the BLA output neurons, resulting in increased excitatory potentials to inhibitory potentials ratio (Rainnie et al. 2004). Somewhat surprisingly, Ucn1-priming in the current study was not accompanied by altered expression of any of the GABA-related genes screened. However, the reduced Sstr2 expression in BLA could explain the reduced

ratio of inhibitory to excitatory potentials following Ucn1-priming. Sstr2 agonists in the BLA directly inhibit BLA-projection neurons and can enhance $GABA_A$ receptor activity (Meis et al. 2005; Muller et al. 2007). Thus, reducing Sstr2 expression may result in reduced inhibitory/GABAergic events in the BLA. Further studies are required to determine whether, following Unc1 priming, neurons projecting from other brain regions differentially regulate BLA output neurons.

Reducing Sstr2 activity in the BLA appears to be anxiogenic and increasing Sstr2 activity appears to delay anxiety-induction. Sstr2 agonist infusion into the BLA blocked anxiogenic-like responses induced by an acute high dose Ucn1 BLA infusion. However, the Sstr2 agonist did not alter anxiety-like measures at baseline, unlike previous reports where Sstr2 i.c.v. infusions inducing anxiolytic-like responses (Engin and Treit 2009). This may suggest that under the current testing conditions anxiety-like behavior was already at a minimum. Alternately, endogenous Sst activity at the Sstr2 in the BLA during baseline conditions may be sufficient to maintain low level of anxiety and BLA activity; an increase in baseline excitation of the BLA network may be needed to see the effects of Sstr2 selective agonists. The Sstr2 and CRFR1, the primary target of Ucn1 in the BLA (Gehlert et al. 2005), are both G-protein coupled receptors with well-known opposing downstream effects; CRFR1 are positively coupled to adenylyl cyclase whereas the Sstr2 are negatively coupled to adenylyl cyclase. Thus, the blockade of acute Ucn1-induced anxiety by a selective Sstr2 agonist may be a result of opposing effects of CRFR1 and Sstr2 on adenylyl cyclase.

Sstr2 agonist pretreatment also blocked or delayed anxiety-like sensitization to Ucn1-priming. However, Sstr2 agonist pretreatment did not prevent the development of a chronic anxiety-like phenotype or the reduction in Sstr2 expression induced by Ucn1-priming. Thus, while treatment with Sstr2 agonist may acutely oppose the anxiogenic effects of acute CRFR1 stimulation in the BLA, the molecular and network changes induced by repeated activation of CRFR1 receptors in the BLA (as likely to be seen in repeated stress episodes) appear to be unaffected. These novel findings have important implications for understanding the effects on transient anxiety observed following isolated stressful events as opposed to the emotional phenotypic alteration that may develop following repeated or prolonged stress. From a therapeutic perspective, this suggests that while Sstr2 agonists may work as putative anxiolytic drugs during acute stress, they may not be effective in preventing long term effects of chronic stress.

There is evidence that i.c.v. infusions of Sstr2 agonists have acute anxiolytic- and antidepressant-like effects (Engin and Treit 2009). Further, one of the key changes observed after chronic imipramine treatment is an upregulation of Sst signaling (Nilsson et al. 2012). This latter finding is particularly important considering that imipramine was the first effective treatment developed for panic disorder (Mavissakalian and Perel 1989) and a panic prone phenotype develops in rats after Ucn1-priming (Sajdyk et al. 1999). The current results support the idea that acute beneficial effects of Sstr2 agonists exist. However, they may not be as effective at ameliorating robust and deleterious effects of chronic stress in the long-term. This idea is consistent with a report where injections of a selective Sstr2 agonist L-054,264 into the amygdala of rats strongly attenuated fear expression but did not affect fear learning (Kahl and Fendt 2014). Perhaps Sstr2 agonists could be useful in combination

with other agents to attenuate negative effects of chronic stress both acutely and for the long-term. Global deletion of the Sst type 4 receptor (Sstr4) increases sensitivity to chronic stress in the BLA (Scheich et al. 2017). Considering that Sstr2 indirectly regulates the Sstr4 in rat models of siezures and memory systems (Aourz et al. 2011; Gastambide et al. 2010), and the Sstr4 gene showed a trend for down regulation following Ucn-1 priming in the current study, a future direction is to investigate a combination of Sstr2 and Sstr4 agonists for anxiolytic effects in this model of chronic anxiety.

The data presented here demonstrate that the persistent anxiety-like phenotype observed with Ucn1-priming in the BLA is associated with a selective reduction of Sstr2 gene expression, and thus mechanistically models a chronic stress response. Future studies should seek to confirm that this decrease in mRNA equates to less functional protein expression. Based on acute pharmacological studies, it is likely that the reduced expression of Sstr2 in the BLA is a key underlying mechanism for the chronic anxiety phenotype observed after Ucn1-priming. Furthermore, although Sstr2 activation in the BLA may be able to block acute anxiogenic effects of stress, it does not appear to suppress the development of long-term consequences of prolonged exposure to stress-related challenges.

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Abbreviations:

BLA	basolateral amygdala
Actb	beta-Actin
BSA	bovine serum albumin
Chrm4	muscarinic receptor 4
Chrna4	cholinergic nicotinic receptor subunit alpha 4
CRFR	corticotropin-releasing factor receptors
CYN	CYN-154806 [Ac-4NO2-Phe-c(DCys-Tyr-DTrp-Lys-Thr-Cys)-D/LTyr-NH2]
DHβE	Dihydro-beta-erythroidine hydrobromide
Inf	infusion
i.c.	intracranially
Ldha	lactate dehydrogenase A
nACh	nicotinic receptor

qRT-PCR	quantitative real-time polymerase chain reaction
RQ	relative quantification
Rpl13a	ribosomal protein L13A
Rplp1	ribosomal protein, large, P1
SI	social interaction
Sst	somatostatin
Sstr2	somatostatin receptor 2
Sst4	somatostatin receptor 4
BIM	Sstr2 agonist, BIM-23027
Ucn1	urocortin 1
Veh	vehicle
CRF	corticotropin-releasing factor

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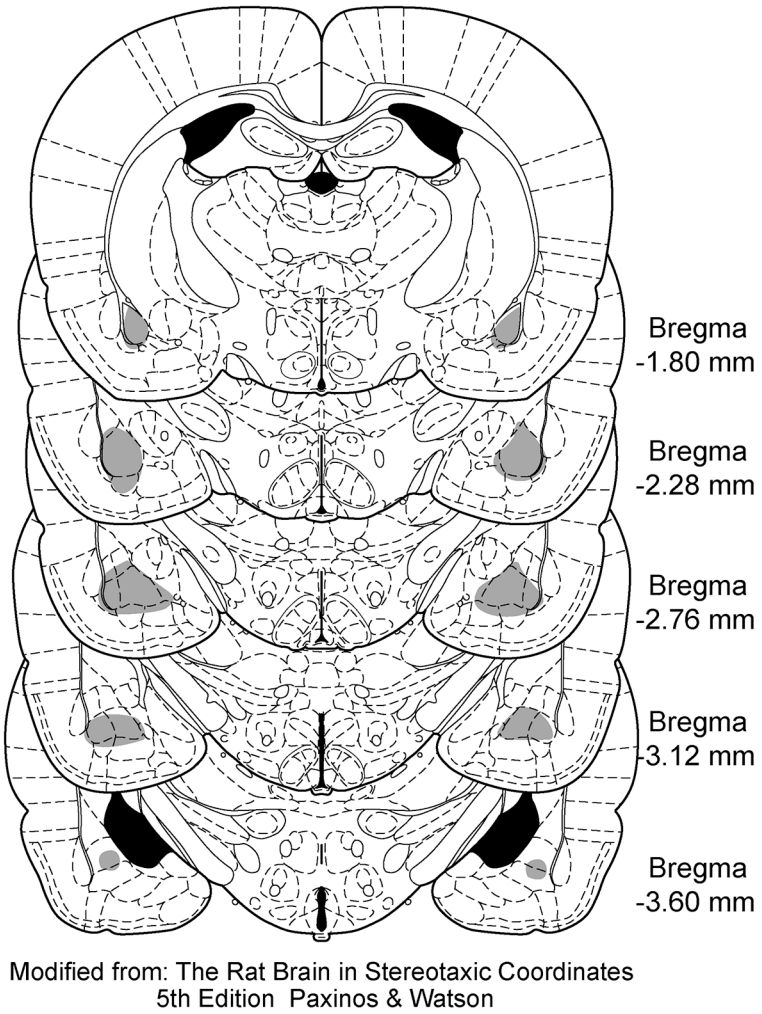

Figure 1: Location of injections.

All rats included in these studies had bilateral injection sites located within the shaded regions. Numbers on the right indicate distance (mm) posterior from bregma.

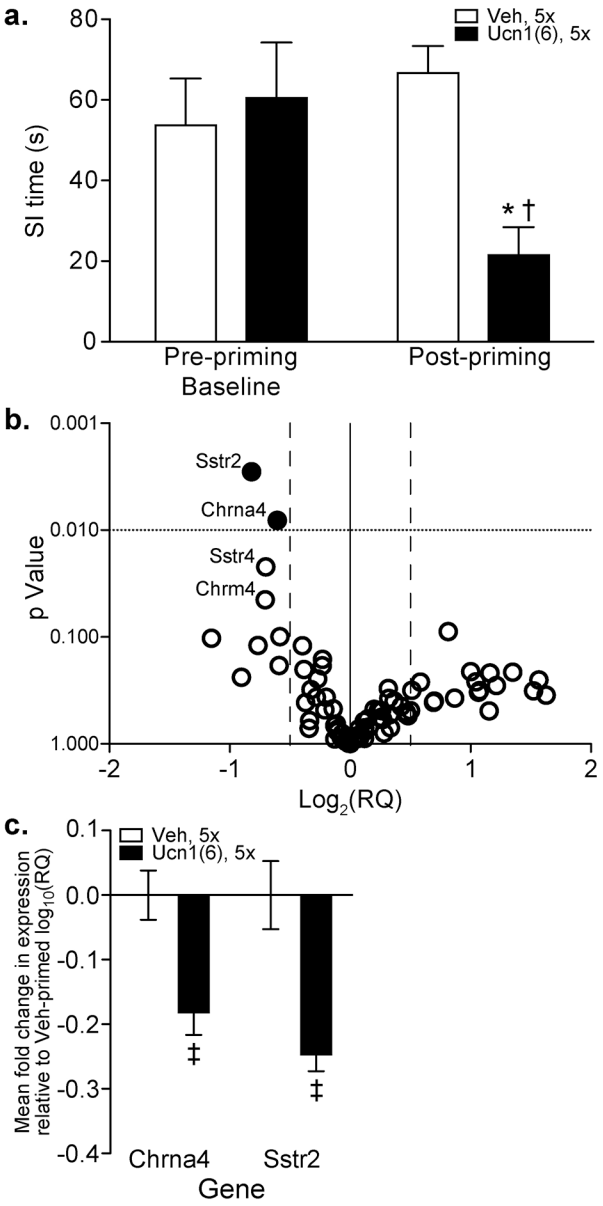

Figure 2. Ucn1-priming induces persistent anxiety-like behavior and selective reduction of gene expression in the BLA.

Presented in (**a**) are SI times (mean ± SEM) prior to and 3 days after 5 days of Ucn1- or veh-priming. Presented in (**b**) is a volcano plot of gene expression from the Neurotransmitter Receptors and Regulators RT^2*Profiler*™ PCR Array. Data presented are mean BLA mRNA expression levels, for each of the 84 target genes, of Ucn1-primed rats relative to veh-primed rats. Data are expressed as ± fold changes (log2, x-axis) plotted against level of significance (p-values, y-axis). Horizontal dotted line represents the assigned cut-off for significance (p value of 0.05), thus values above the dotted line are significantly different from vehicle values. The dashed vertical lines represent a ±1.5 fold change in expression. Closed circles are genes that met the criteria for genes of interest and open circles are genes that did not. Data presented in (**c**) are mean ± SEM relative expression, of Ucn1-primed (solid

bars) and veh-primed rats (open bars, all at zero), for the genes of interest. Here data are plotted as $\log_{10}$(RQ) where RQ is the fold change in gene expression relative to vehicle primed rats determined by the delta delta Ct method; Ucn1-primed (n=5), Veh-primed (n=4). Abreviations: Sstr2, somatostatin receptor 2; Chrna4, nicotinic receptor alpha4; Sstr4, somatostatin receptor 4; Chrm4, muscarinic receptor 4;Ucn, urocortin 1; Veh, vehicle. Significance, * indicates significantly different from baseline; † indicates significantly different from the vehicle-primed group.

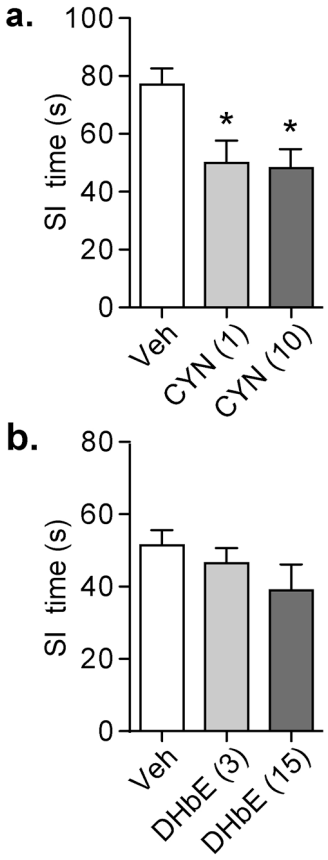

Figure 3. Selective antagonist infusions into the BLA and anxiety-like behavior.

(a) Selective Sstr2 antagonist infusion into the BLA induce anxiety-like behavior. Intra-BLA infusions of Sstr2 antagonist, CYN-154806 [(CYN), 1 or 10 pmol], significantly reduced SI time (mean±SEM) compared to vehicle infusion. * indicates significantly different from vehicle.

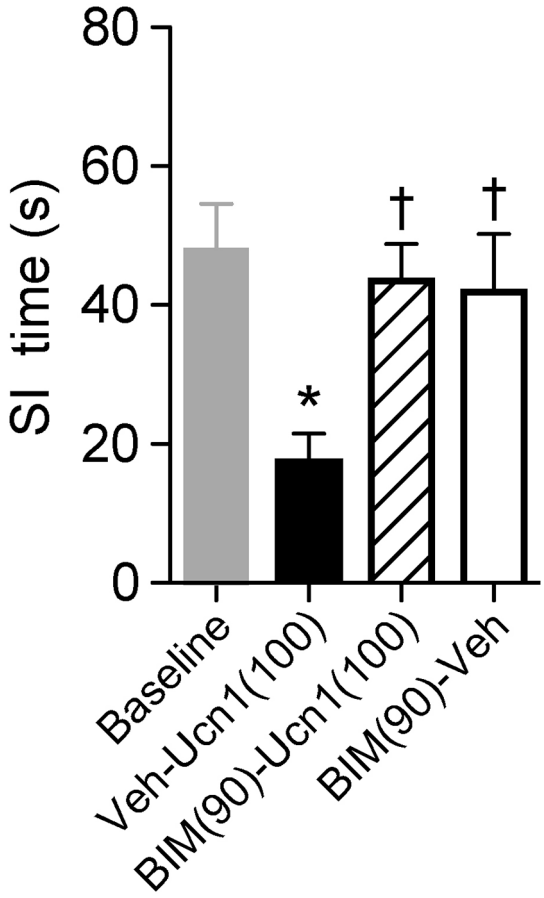

Figure 4. Pretreatment with Sstr2 agonist in the BLA blocks anxiety-like behavior induced by acute Ucn1 in the BLA.

Presented here are SI times (mean ± SEM) of rats at baseline (no treatment) or following one of three intra BLA treatment conditions: Vehicle infusion followed by infusions of 100 fmol of Ucn1 [Veh-Ucn1(100); black bar]; infusion of the Sstr2 agonist, BIM-23027 (90 pmol) followed by infusion of 100 fmol of Ucn1 [BIM(90)-Ucn1(100); striped bar] and BIM(90) followed by veh infusions [BIM(90)-Veh; white bar]. * indicates significantly different from baseline; † indicates significantly different from Veh-Unc1(100).

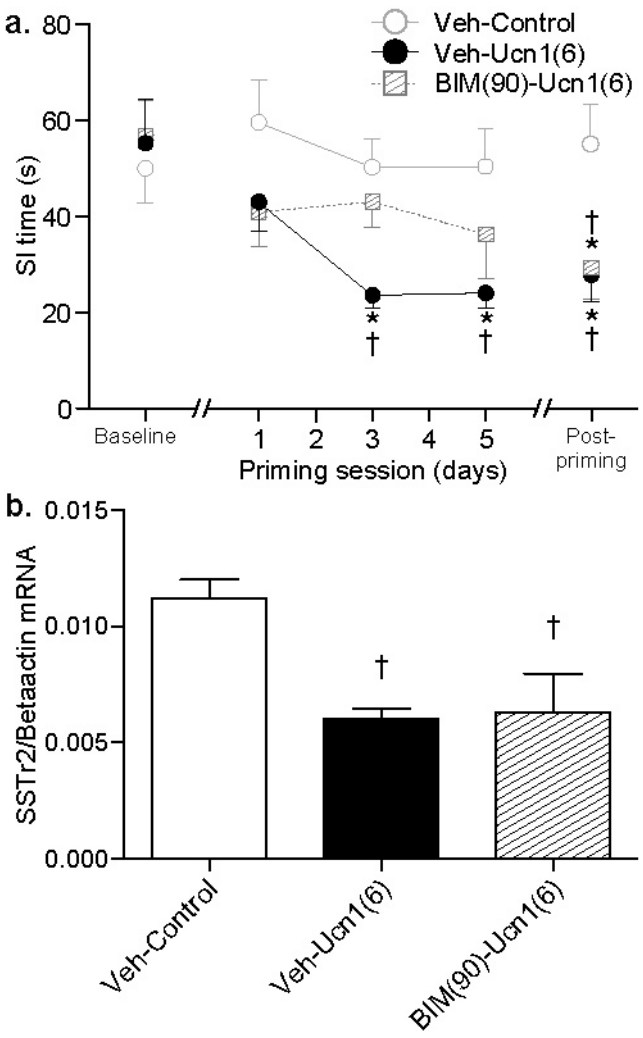

Figure 5. Pretreatment with Sstr2 agonist during Ucn1-priming does not prevent the development of a persistent anxiety-like phenotype or reduced expression of Sstr2 gene.

Presented in **(a)** are SI times (mean ± SEM) of rats from 3 intra-BLA priming conditions: open circles represent control-priming (vehicle) with vehicle pretreatment (Veh-control); closed circles represent Ucn1-priming (6 fmol) with vehicle pretreatment [Veh-Ucn1(6)] and striped squares represent Ucn1-priming with BIM-23027 (90 pmol) pretreatment [Bim(90)-Ucn1(6)]. All treatments were delived bilaterally into the BLA for five consecutive days. Pretreatments were delivered 30 min prior to treatments. Rats received SI testing 72 hr prior to the 1st priming session (Baseline), 30 min after priming on days 1, 3 and 5 and 72 hours after the last priming session (Post-priming, day). Presented in **(b)** are expression levels (mean ± SEM) of Sstr2 gene from the BLAs of the rats in (a). Data are presented as the ratio of Sstr2:Beta actin expression as determined by absolute qRT-PCR. * indicates significantly different from baseline, † indicates significantly different from Veh-control group.

Table 1.

Genes included in the Neurotransmitter Receptors and Regulators RT^2 *Profiler*™ PCR Array arranged by class and function.

Class	Receptor / rec. subunit genes	Biosynthesis, catabolism, anchoring, transport genes
Inhibitory amino acids	*GABA*: Gabra1, Gabra2, Gabra3, Gabra4, Gabra5, Gabra6, Gabrb2, Gabrb3, Gabrd, Gabre, Gabrg1, Gabrg2, Gabrp, Gabrq, Gabrr1, Gabrr2, *Glycine*: Glra1, Glra2, Glra3, Glrb	Gad1, Gad2, Abat
Catecholamines	*Dopamine*: Drd1a, Drd2, Drd3, Drd4, Drd5 *Serotonin*: Htr3a	Comt, Maoa, Th
Cholinergic	*Nicotinic*: Chrna1, Chrna2, Chrna3, Chrna4, Chrna5, Chrna6, Chrnb1, Chrnb2, Chrnb3, Chrnb4, Chrnd, Chrne, Chrng *Muscarinic*: Chrm1, Chrm2, Chrm3, Chrm4, Chrm5	Chat, Prima1, Anxa9
Neuropeptides	*CCK*: Ccka, Cckb *Galanin*: Galr1, Galr2, Galr3 *neuropeptide FF*: Npffr1, Npffr2 *NPY*: Npy1r, Npy2r, Npy5r, Ppyr1, Gpr83, Prokr1, Prokr2 *Somatostatin*: Sstr1, Sstr2, Sstr3, Sstr4, Sstr5 *Tachykinin*: Tacr1, Tacr2, Tacr3 *Other* *(26RFa*, Adrenocorticotropin, Bombesin, Neuromedin U, prolactin releasing hormone): Gpr103 (Qrfpr), Mc2r, Brs3, Grpr Nmur1, Nmur2	

Table 2:

Designed real-time primers used for cloning Somatostatin 2 receptor or beta Actin.

Name	Primer	Sequence	Product length
beta Actin	Forward	5’-GAAGATCAAGATCATTGCTCCTCC-3’	approx. 200 bp
	Reverse	5’-TTTTCTGCGCAAGTTAGGTTTTGTC-3’	
Somatostatin 2 receptor	Forward	5'-TATCCTCACCTACGCCAACAGCT-3’	approx. 180 bp
	Reverse	5'-CTCTGGGTCTCCGTGGTCTCATT-3’	