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Anxiety-like behavior is modulated by a discrete subpopulation of interneurons in the basolateral amygdala

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Abstract

The basolateral amygdala (BL) is a putative site for regulating anxiety, where inhibition and excitation respectively lead to decreases and increases in anxiety-like behaviors. The BL contains local networks of GABAergic interneurons that are subdivided into classes based on neurochemical content, and are hypothesized to regulate unique functional responses of local glutamatergic projection neurons. Recently it was demonstrated that lesioning a portion of the BL interneuronal population, those interneurons that express neurokinin1 receptors (NK_{1r}), resulted in anxiety-like behavior. In the current study, these NK_{1r} expressing cells of the BL are further phenotypically characterized, demonstrating approximately 80% co-expression with GABA thus confirming them as GABAergic interneurons. These NK_{1r} interneurons also co-localize with two distinct populations of BL interneurons as defined by the neuropeptide content. 41.8% of the NK_{1r} positive cells are also positive for neuropeptide Y (NPY) and 39.7% of the NK_{1r} positive cells are also positive for cholecystokinin (CCK). In addition to enhancing the phenotypic characterization, the extent to which the NK_{1r} cells of amygdala nuclei contribute to anxiety-like responses was also investigated. Lesioning the NK_{1r} expressing interneurons, with a stable form of substance P (SSP; the natural ligand for NK_{1r}) coupled to the targeted toxin saporin (SAP), in the anterior and posterior divisions of the BL was correlated to increased anxiety-like behaviors compared to baseline and control treated rats. Furthermore the phenotypic and regional selectivity of the lesions was also confirmed.

Keywords

Substance P; Saporin; NPY; CCK; GABA

The basolateral nucleus of the amygdala (BL) is a putative site of emotional memory and regulation of anxiety (Davis, 1994, McGaugh et al., 2002), where inhibition decreases, and excitation increases anxiety-like behaviors (Sajdyk and Shekhar, 1997b). For example, glutamate antagonists in the BL will block both the acquisition and expression of

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conditioned fear in rats (Miserendino et al., 1990, Kim and McGaugh, 1992, Sajdyk and Shekhar, 1997b). Benzodiazepines, which positively modulate the GABA_A receptor, are anxiolytic when injected directly into the BL, whereas blocking the benzodiazepine site on the GABA_A receptor in the BL blocks anxiolytic effects of systemically administered benzodiazepines (Sanders and Shekhar, 1995a). Conversely, injections of GABA_A receptor antagonists into the BL results in experimental ‘anxiety’ as assessed in the social interaction (SI), conflict test and conditioned place avoidance (Sanders and Shekhar, 1991, Sanders and Shekhar, 1995a, Sanders and Shekhar, 1995b, Thielen and Shekhar, 2002), through disinhibition of glutamate signaling (Rainnie et al., 1991a, Rainnie et al., 1991b, Sajdyk and Shekhar, 1997a, Sajdyk and Shekhar, 1997b).

Anatomical and histochemical studies have described the BL as a cortex-like structure (McDonald, 2003) that has glutamatergic principal cells and GABAergic interneurons. BL interneurons, which constitute only 15% of the total neuronal population in the BL (McDonald, 1982, McDonald, 1992), exist as a network of distinct classes based on calcium binding protein and neuropeptide content (McDonald and Betette, 2001, McDonald and Mascagni, 2001, McDonald and Mascagni, 2002, Mascagni and McDonald, 2003, Muller et al., 2003), as observed in cortical interneurons. Electrophysiological studies have demonstrated that BL interneurons determine the state of excitability of the BL principal neurons (Rainnie et al., 1991b, Gean and Chang, 1992, Smith and Dudek, 1996, Mahanty and Sah, 1998, Rainnie, 1999), and thus are likely to be critical in regulating expression of anxiety-like behaviors.

Like cortical interneurons, different classes of BL-interneurons have been hypothesized to have unique functional roles, including discriminate regulation of neuronal firing patterns and regulation of behaviors, as well as specific roles in pathologies such as epilepsy and anxiety (Tuunanen et al., 1997, Freund, 2003). Recently, we demonstrated that lesioning of the BL with the targeted toxin SSP-SAP, a toxin that selectively lesions neurons which express neurokinin1 receptors (NK_{1R}), increases anxiety-like behaviors in rats (Truitt et al., 2007). It has been previously demonstrated that NK_{1R} in the BL co-localize with interneurons containing NPY and somatostatin (SOM) as well as calbindin (CB), but do not overlap with parvalbumin (PV) or calretinin (CR) containing interneurons (Levita et al., 2003). However, the amount of co-localization with GABA and cholecystikinin (CCK) has thus far only been inferred and not systematically investigated. Here we present a detailed study of the phenotypic profile of the neurons that are lesioned by SSP-SAP injections into the BL and its effects on different anxiety-like behaviors. Furthermore, we conducted correlative studies to determine the anxiety-like behavior predicted by the extent of lesions within the different nuclei of the amygdala, as well as the extent of loss of GABA and NPY interneurons in the BL.

Experimental procedures

Animals—Male Wistar rats (Harlan Sprague-Dawley, Indianapolis, IN) between 325–350 g were individually housed with free access to food and water. The room temperature was maintained at 72°F (22°C) on a 12-h light, 12-h dark schedule. All studies were conducted in accordance with the *NIH Guidelines for the Care and Use of Laboratory Animals* (NIH Publication no. 80–23) revised 1996 and the guidelines of the IUPUI Institutional Animal Care and Use Committee. Twenty-eight rats, n=4 for the dual immunofluorescent experiments, n=12 for SI experiments and n=10 for EPM experiments, were used in total.

Targeted toxin administration—All surgeries were conducted 1 – 4 days following baseline SI testing. Prior to surgery, rats were anesthetized with IsoFlurane© by removing them from their home cages and placing them in a closed plexiglass box which was

connected to an isoFlurane system (MGX Research Machine; Vetamic, Rossville IN). They were placed into a stereotaxic instrument (Kopf Instruments, Tujunga, CA) with the incisor bar set at -3.3 mm and attached to a nose cone connected to IsoFlurane© system during the surgeries. Two stainless steel injectors (33 gauge, 10 mm length) placed inside guide cannulae (28 gauge, cut to 2mm length, Plastic Products, Roanoke, VA) were situated into guide cannulae holders fixed onto the stereotaxic arms. The injectors were connected to Hamilton syringe pump (Harvard Apparatus) set to a flow rate of $0.25 \mu\text{l}/\text{min}$. The guide cannulae were lowered into position of targeting the BL for a series of 6 bilateral injections. Two injections (separated by 0.5 mm in depth) were delivered at each of the three anterior-posterior (AP) coordinates [AP(1), -2.2 ; M, ± 5.0 ; V, -8.4 , -8.9 ; AP(2), -2.6 , M, ± 5.0 ; V, -8.4 , -8.9 ; and AP(3), -3.0 , M, ± 4.8 ; V, -8.6 , -9.2] using a standard stereotaxic atlas of the adult rat brain [i.e., Paxinos and Watson (1986)]. Each injection was administered over 2 min periods (total volume of $0.5 \mu\text{l}$) and the injector was left in place for an additional 2 min following completion of injection when moving to the next anterior position. The movement of a bubble in the tubing monitored delivery to ensure accurate volume was delivered. Rats received injections of either the targeted toxin SSP-SAP ($4.0 \text{ ng}/0.5 \mu\text{l}$), which consists of the ribosome inhibitor saporin (SAP) conjugated to an analogue of substance P (SSP), or blank-SAP ($4.0 \text{ ng}/0.5 \mu\text{l}$), which consists of the ribosome inhibitor saporin conjugated to the 11 amino acids in SSP with a jumbled sequence with no known affinity for membrane targets and thus unable to enter cells. Following surgeries, rats were given 7 to 10 days to recover, during which time the health status of the animals were monitored daily.

Behavioral Tests

Social interaction test (SI)—Anxiety-like behavior was measured utilizing a modified version (Sanders and Shekhar, 1995b) of the SI test (File, 1980). Here the treated animal was simultaneously placed into the SI box (0.9 m long $\times$ 0.9 m wide with walls 0.3 m and an open top) with an age, sex and weight matched partner rat. Partner rats are untreated and did not undergo any surgeries. The testing is carried out under low light (40 watt red lighting) and familiar conditions. All testing is videotaped via a camera mounted on the ceiling directly above the SI box. Scoring of SI times are determined as previously described (Sajdyk and Shekhar, 1997b). Seven days after arrival in colony, rats were assigned to a treatment group, either SSP-SAP ($n=6$) or blank-SAP ($n=6$), and baseline social interaction (SI) times were recorded. Following surgery and a 7 to 10 day recovery period, rats were again tested in the SI test with a novel partner rat. This procedure was repeated again with novel partner rats each time at 3 weeks post surgery and 6 weeks post surgery.

Elevated plus-maze—Experimental “anxiety” was assessed in a separate group of rats, treated with SSP-SAP ($n=5$) or blank-SAP ($n=5$) as described above, in the elevated plus maze (EPM). Here the treated animal was placed into the center area of the plus-maze and the total amount of time (in sec) spent in the open arms, closed arms and central decision area was recorded during the 5-min test period. Additionally, the number of entries into these areas was also recorded..

Perfusion and tissue processing

Rats were anesthetized with IsoFlurane© and perfused transcardially with 100 ml 0.9% saline, followed by 500 ml of 4% paraformaldehyde in 0.1 M PBS (pH $7.3-7.4$). Brains were then removed and post-fixed for 1 hr at room temperature in the fixative. Brains were then placed in cryoprotectant (30% sucrose in 0.1 M PB) for an additional 4–5 days. Coronal brain sections were cut at $35 \mu\text{m}$ (Microm freezing microtome, Richard Allen, Kalamazoo, MI) and collected in four parallel series in cryoprotectant solution [30% sucrose, 30% ethylene glycol in 0.1 M PB; (Watson et al., 1986)].

Immunocytochemistry

Immunoperoxidase reactions—All incubations of brain sections were performed at room temperature with gentle agitation. A series of brain sections were extensively rinsed in phosphate buffered saline (0.1M, PBS) between incubations. Free-floating sections were blocked with 1% H₂O₂ for 10 min at room temperature and then soaked for one hour in incubation solution (PBS containing 0.1% bovine serum albumin and 0.4% triton X-100). Next, sections were incubated overnight with a primary antiserum (either rabbit anti-NK1r, 1:2000 dilution, Advanced targeting system, San Diego CA; mouse anti-NeuN 1:10,000, Chemicon, Billerica MA; or rabbit anti-CCK-8, 1:10,000 dilution, Immunostar, Hudson WI). Subsequently, sections were exposed for 60 min to the appropriate biotin-conjugated IgG, (goat anti-rabbit or goat anti mouse 1:500 dilution in incubation solution, Vector Laboratories, Burlingame, CA) for 60 min and to avidin-biotin-horseradish peroxidase (ABC-elite, 1:1,000 dilution in PBS; Vector Laboratories). The peroxidase complexes were visualized by exposure for 10 min to a chromogen solution containing 0.02% 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma, St. Louis MO) with or without 0.02% nickel sulfate in 0.1 M PB with hydrogen peroxide (0.015%) to produce a brown (NeuN and CCK-8 staining) or a blue-black (nickel enhancement, NK1r staining) reaction product. Extensive washing in 0.1M PB terminated the reaction. Sections were then mounted on superfrost plus glass slides (Fisher Scientific, Hampton NH) and cover-slipped with DPX (Electron Microscopy Sciences, Fort Washington, PA). Immunocytochemical controls included pre-incubation with blocking peptides and omission of primary antibodies.

Dual Immunofluorescence—Following extensive rinsing, free-floating sections were blocked for one hour in incubation solution (0.1 M PBS containing 0.1% bovine serum albumin and 0.4% triton X-100). Next, sections were incubated overnight with a primary antiserum (rabbit anti-NK1r, 1:800 dilution). Subsequently, sections were exposed for 60 min to goat anti-rabbit biotin-conjugated IgG, (1:500 dilution in incubation solution, Vector Laboratories) and for 60 min to avidin-biotin-horseradish peroxidase (ABC-elite, 1:1,000 dilution in PBS; Vector Laboratories). Next the sections were exposed to tyramide amplification solution (1:250 dilution; PerkinElmer, Waltham MA), and to DTAF-conjugated streptavidin (1:100 dilution; Jackson ImmunoResearch, West Grove PA, for GABA and CCK-8 dual-labeling) or Cy3-conjugated streptavidin (1:100 dilution; Jackson ImmunoResearch, for NPY dual-labeling). Subsequently, sections were incubated overnight with the second primary antiserum (either rabbit anti-CCK-8, 1:1000 dilution, ImmunoStar; rabbit anti-GABA 1:1000 dilution, Sigma; or sheep anti-NPY, 1:1500 dilution, PenLabs, Torrance CA), then incubated with CY3-conjugated donkey-anti-rabbit (Jackson ImmunoResearch, 1:100 dilution) or donkey anti-sheep Alexa488 (1:200 dilution, Molecular Probes, Carlsbad CA). Sections were then washed, mounted on plus charged glass slides, air-dried, and cover-slipped with (Gelvatol) containing an anti fading agent (1,4-diazabicyclo (2,2)octane; Sigma). To rule out cross reactivity, staining occurred as described above except that the second primary incubation was omitted in control sections. No second label was observed in these sections.

Cell counts

Slides were coded so that analysis could be performed blindly.

Counts for light microscopy—For each brain, serial coronal sections cut at 35 μ m (distance between sections 140 μ m prior to dehydration) were assessed for NK1r-IR or NeuN-IR. Only sections of the amygdala inclusive to the AP injection target sites were counted; this included sections between -2.2mm bregma to -3.0 mm bregma (Paxinos G, 1986). Overall, approximately four to five sections/brain were included in the analysis. The analyses were performed using an Olympus BX51 light microscope, with a motorized stage

connected to a computer equipped with Stereo Investigator Software analytical software (Microbrightfield, Inc., Williston, VT, USA). Utilizing this software and computerized control of the microscopes stage, amygdalar nuclei borders were traced at 4x objective. The tracing was performed first on the Neu-N- stained tissue using images from the rat brain atlas of Paxinos (1986) to confirm the cytoarchitecture. A combination of the atlas images and Neu-N-stained tissue were used to assist tracing the amygdalar borders in the NK1r-stained sections (adjacent sections to the Neu-N stained sections). For each amygdalar nuclei, at each level bilateral counts of immunoreactive cells were performed within the given tracings at 40x objective using the optical fractionator setting of the Stereo Investigator Software. The optical fractionator grid size was set equal to the counting frames ($375\ \mu\text{m} \times 300\ \mu\text{m}$) so that all cells were counted in each nucleus at each level. This method of counting was used because of the low number and uneven distribution of NK1r-IR cells within the amygdalar nuclei. For consistency this counting method was employed in the NeuN-IR cell counts as well. The total cells counted per nuclei at all sections were divided by the total volume of each section counted for each region. Total volumes from the sections counted for each region were calculated by the sum of area (determined by the optical fractionator) $\times$ depth (set at $15\ \mu\text{m}$) for each section. Data is reported as number of cells per mm^3 . Estimates for the total number of NK_{1r} cells throughout the regions counted were determined by the Stereo Investigator (Optical Fractionator) software.

Immunofluorescent counts—Cell counts for phenotypic characterization of BL interneurons were performed on brains from control rats receiving no injections. The cell counts were performed within the BL one color images (red or green filter) at a time resulting in two set of counts, one for NK_{1r}-IR and the other the interneuronal marker. To keep the areas consistent between counts using different filters, a transparent overlay was taped onto the monitor displaying the fluorescent images. The filters were removed and the transmitted light image was displayed. From this image, the borders of the BL were traced onto the overlay with a marker. The stage was not moved during this process. The proper fluorescent filters were then put back in place and the number of fluorescent cells displayed within the borders of the BL counted. The filter was changed and the process repeated. In order to keep the borders true during the counts, only the focal plane was adjusted while counting. Cells counts for colocalization of markers were determined from merged images using a SPOT camera and SPOT v4 software. Fluorescent cell counts to determine the extent of lesion on NK_{1r}-IR and GABA-IR or NPY-IR cells were performed on serial sections collected from the same brains used for the light microscopy study described above. Here, the counts were performed similar to the method described for the light microscopy study except that cell counts were performed on merged images captured from a SPOT camera and assisted by SPOT v4 software. Areas were calculated using SPOT v4 software and volume for each slide was estimated using $35\ \mu\text{m}$ as a depth for each section since the sections were not dehydrated. Thus data is also presented as cells/ mm^3 . To determine the CCK large (CCK_L) and CCK small (CCK_S) the soma of the fluorescently labeled cells were measured from photomicrographs using the SPOT software line distance tool.

Optical density measures—For analysis of optical density of NK_{1r}-IR and NPY-IR, images were captured at 20x magnification using a light microscope and SPOT CCD-camera. The images were centered within the region of interest (CeA for the NK_{1r}-IR density study and the BL for the NPY-IR density study). Images were imported into NIH-imageJ analysis software and the same threshold was applied to all images. Numbers of pixels above threshold were determined for each animal and group averages were calculated.

Data analysis and statistical methods

The SI times of the rats were compared using repeated measures ANOVA with SSP-SAP v. Blank-SAP treatment as main effect and the testing day as the repeated measure. In the case of significance with the ANOVA, post-hoc comparisons were made using the Tukey's test to compare between groups and a Dunnett's test was performed to compare within subjects to baseline values with an alpha of 0.05. EPM values (means of percentage of test time spent in open arms and percentage of entries that were into the open arms) and cells counts were compared between treatment groups using a student's t-test with an alpha of 0.05. Additionally, a one-way repeated measures ANOVA was used for cell counts when comparing multiple regions between treatment groups, with the main effect being treatment and the repeated measure being regions of interest. When appropriate, a Tukey's test, with an alpha of 0.05, was performed to compare groups.

Results

Phenotypic characterization of BL interneurons

It has been reported that NK_{1r} expressing cells of the BL co-localize with interneurons containing NPY, SOM and CB, but do not overlap with PV or CR containing interneurons. However, NK_{1r} co-expression with GABA and CCK within the same interneurons has not been examined. Here we used dual labeling immunofluorescence to determine the extent to which NK_{1r}-IR cells were also GABA-IR, CCK_L-IR [defined as large (L) CCK-IR cells with a soma diameter > 10 µm (Mascagni and McDonald, 2003)], and NPY-IR within the BL (Figure 1A). GABA-IR was observed in 78.3% of the cells with NK_{1r}-IR in the BL while only 2.8% of the GABA-IR cells in the BL had NK_{1r}-IR (Table 1). Some NK_{1r}-IR cells also had CCK8-IR. 39.7% of NK_{1r}-IR cells in the BL also had CCK_L-IR. Conversely, 48.9% of CCK_L cells and 0% of small CCK (CCK_S) cells in the BL [soma diameter < 10 µm (Mascagni and McDonald, 2003)] had NK_{1r}-IR (Table 1). Additionally, NPY-IR and NK_{1r}-IR was also observed in the same cells within the BL. Similar to what has been previously described (Levita et al., 2003), the vast majority of NPY-IR cells in BL (88.8%) also had NK_{1r}-IR. Conversely, 41.8% of the NK_{1r}-IR cells in the BL had NPY-IR. Mascagni and McDonald (2003) demonstrated that the CCK_L and NPY represent two different and exclusive sub populations of BL interneurons. Thus, these two neuropeptide markers would account for majority of NK_{1r} expressing interneurons in the BL (see Figure 1B for diagram). In all rats the number of neuropeptide- and GABA -IR cells may be underestimations of actual number of cells that express these markers since colchicine treatment, which would stop axonal transport, was not employed.

Histological effects of SSP-SAP injections into the BL

SSP-SAP injections produced regionally selective lesions—SSP-SAP injections lesioned NK_{1r} cells compared to blank-SAP injections (see Figure 2A and B for example and of region of interest). The reduction in NK_{1r}-IR cells by SSP-SAP injections was primarily selective to the BL, including both BL_a and BL_p, however in 3 of 6 SSP-SAP rats the lesions spread to include the LA. In the BL_a and BL_p, the number of NK_{1r}-IR cells were significantly reduced in the SSP-SAP group (n=6) compared to blank controls (n=6); this effect was not evident in either LA or CeA [Figure 3A, repeated measures ANOVA (treatment × region interaction $F_{3,40} = 6.67$, $p < 0.001$, Tukey's $q = 3.22$, $p < 0.05$)]. The Stereo Investigator, optical fractionator software was used to make estimates of the total number of NK_{1r} cells in each region by treatment. These totals are: BL_a – blank-SAP = 299.5 (mean) ± 43.13 (SEM) cells, SSP-SAP = 85 ± 15.9 a 71.5% reduction, BL_p – blank-SAP = 334.1 ± 44.6, SSP-SAP = 137.7 ± 13.8 a 58.8% reduction, LA – blank-SAP = 384.5 ± 88.7 cells, SSP-SAP = 219.3 ± 39.0 a 43% reduction, CeA – Blank-SAP = 492.2 ± 32.3, SSP-SAP = 582.81.6 an increase of 18%. The lesions of BL NK_{1r} cells, induced by SSP-

SAP injections did not reduce NK_{1r}-IR fibers density of the adjacent CeA when compared to rats injected with blank-SAP, further suggesting the lesions were isolated to the BL [Figure 3 inset; measured as pixels above threshold, between groups (Student's $t_{11,2} = 0.23$, $p = 0.82$)]. The SSP-SAP injections, compared to control, also did not lead to non-specific damage in any amygdalar nuclei examined as indicated by no reduction in overall neuronal staining [measured by counting NeuN-IR cells, (Figure 3B, repeated measures ANOVA (treatment $\times$ region interaction $F_{3,40} = 1.04$, $p = 0.39$)]. In these nuclei, not surprisingly, there was a main effect of region on cell density ($F_{3,40} = 66.03$, $p < 0.0001$). Here the neuronal density (NeuN-IR/mm³) in all regions were significantly different from one another except for LA and the BLA, with the CeA containing significantly greater neuronal density and the BLp containing significantly lower neuronal density than all other regions (Tukey's $q = 7.40$; $p < 0.001$ for all regions). Regional volumes used in the above analysis were not effected by treatment (Figure 3c, $F_{3,40} = 1.60$, $p = 0.21$), but there was a main effect of region ($F_{3,40} = 95.13$, $p < 0.0001$). All regions differed in volume except the BLA and BLp (Tukey's $q = 6.02$; $p < 0.01$ for all regions).

SSP-SAP injections produced phenotypically selective lesions—Since, the interneurons that express NK_{1r} have been shown to be a subset of the GABA interneurons and encompass the NPY interneuronal population, the effects of SSP-SAP lesions were assessed in GABA-IR and NPY-IR cells. Since it was observed that NK_{1r} cells were lesioned in the both BLA and BLp these regions were combined for the analysis. The number of NK_{1r}-IR cells in the BL (BLA and BLp combined), from the same rats used in figure 3, was significantly reduced in the SSP-SAP injected rats compared to Blank-SAP injected rats (Figure 4A, $t_{11,2} = 5.31$, $p = 0.0003$). In addition, the NK_{1r}-IR cell lesions were not a result of nonspecific neuronal damage. No significant difference was observed in the number of the neuronal marker NeuN-IR cells in the BL between treatment groups (Figure 4B, $t_{11,2} = 0.967$, $p = 0.359$).

In a subset of the rats ($n = 5$ per group), used in figure 3, the effect of SSP-SAP injections on phenotypically characterized BL interneurons was examined. Here we used dual labeled immunofluorescence for GABA-IR and NK_{1r}-IR and observed a significant reduction in NK_{1r}-IR cells in the SSP-SAP rats compared to Blank-SAP rats ($t_{8,2} = 3.74$, $p = 0.006$, Figure 4C). Additionally, the number of BL interneurons that co-expressed NK_{1r} and GABA were also significantly reduced in the SSP-SAP group compared to the Blank-SAP group (Figure 4C, $t_{8,2} = 3.21$, $p = 0.013$). This lesion of NK_{1r}-IR cells did not, however, significantly reduce the overall number of GABA-IR cells in the BL (Figure 4C, $t_{8,2} = 0.091$, $p = 0.930$) suggesting the toxin did not disrupt the overall population of GABAergic interneurons but rather affected only a specific sub-population of interneurons. Adjacent tissue from the above rats was also stained for NK_{1r} and NPY dual immunofluorescence. Here, as before, the number of NK_{1r}-IR cells was significantly lower in the SSP-SAP rats compared to the Blank-SAP rats ($t_{9,2} = 3.99$, $p = 0.0026$; see Figure 3D). Additionally, the numbers of NPY-IR cells and NPY/NK_{1r}-IR cells were significantly reduced in the SSP-SAP treated rats compared to Blank-SAP rats ($t_{9,2} = 2.74$, $p = 0.021$ and $t_{9,2} = 2.90$, $p = 0.016$, respectively). Interestingly, NPY-IR fibers of the BL were not altered by SSP-SAP injections, as no significant difference was observed in the mean number of pixels above threshold between treatment groups (Figure 3E and F, $t_{9,2} = 0.67$, $p = 0.519$).

Lesions of NK_{1r}-IR interneurons in the BL increased anxiety-like behaviors

Social Interaction—The rats with lesions of NK_{1r}-IR containing interneurons in the BL (SSP-SAP, same rats from figures 3 and 4) displayed increases in experimental “anxiety” behaviors as measured by the SI test (Figure 5). Mean SI time in SSP-SAP ($n=6$) injected rats, but not Blank-SAP ($n=6$) injected rats was significantly reduced 1 week (7 – 10 days)

following lesion surgery compared to baseline (ANOVA, $F = 25.62$, $p = 0.0004$; Dunnett's $q = 6.05$, $p < 0.001$); additionally the mean SI time, following injections, was significantly lower in the SSP-SAP rats compared to Blank-SAP rats (Tukey's $q = 5.29$, $p < 0.001$). This reduction in mean SI time remained significantly lower in the SSP-SAP rats compared to baseline and blank-SAP rats at 1, 3, and 6 weeks post injection (within Dunnett's $q = 6.05$, $p < 0.01$; between Tukey's $q = 5.15$, $p < 0.01$), suggesting a lasting effect. No reductions in SI times were observed in the Blank-SAP rats following surgery. No obvious motor impairments were observed in any rats examined.

Additionally, the correlation between SI times and the number of NK_{1r}-IR cells in the amygdalar nuclei were assessed (Figure 5B–E). Here, post treatment SI times (week 1) and overall NK_{1r}-IR cell densities in the basal amygdaloid complex (including the BLA, BLp and the LA) correlated significantly (Pearson's $r = 0.819$, $p = 0.0011$, $n = 6/\text{group}$, data not shown). When looking specifically at the nuclei that comprise the basolateral complex only the BLA and BLp significantly and positively correlate with week 1 SI time (BLA, Pearson's $r = 0.870$, $p = 0.0002$; BLp, Pearson's $r = 0.795$, $p = 0.002$). Numbers of NK_{1r}-IR cells remaining in the LA and week 1 SI time reached only trend (Pearson's $r = 0.540$, $p = 0.0699$). However, post treatment SI times clearly did not correlate with number of NK_{1r}-IR cells in the adjacent CeA nucleus (Pearson's $r = -0.167$, $p = 0.604$). Combining the counts of the BLA and BLp into a single for the BL value (as was done in figure 3) strengthen the correlation between NK_{1r}-IR and week one SI time ($r = 0.874$, $p = 0.0002$, data not shown). Taken together, these data suggest that increases in anxiety-like behaviors observed in the SI test are likely a result of lesioning NK_{1r} interneurons in the BL, and less likely a result of lesions in adjacent regions, however this later statement was not systematically investigated. Additionally, correlations between week one SI times and BL cells counted in the dual immunofluorescences studies were also analyzed. Here it was found the GABA-IR cells/ mm^3 of the BL were not correlated with week 1 SI times ($r = 0.473$, $p = 0.284$) but there was a significant correlation for week 1 SI time and all BL cells immunoreactive with NK_{1r} as well as the cells immunoreactive for both NK_{1r} and GABA ($r = 0.770$, $p = 0.043$ for both NK_{1r} and co localized counts, data not shown). In the BL NK_{1r}-IR, NPY-IR and co localized cells/ mm^3 all significantly correlated with week 1 SI time ($r = 0.862$, $p = 0.0028$, $r = 0.789$, $p = 0.012$, and $r = 0.749$, $p = 0.020$ respectively; data not shown).

Elevated Plus Maze—To confirm the anxiogenic-like affect of NK_{1r}-IR cell lesions in the BL, the effect of ablating the NK_{1r}-IR interneurons in the BL on EPM behavior was evaluated in a separate set of rats. As observed in the SI experiment, rats treated with SSP-SAP demonstrated increased anxiety-like behavior in the EPM compared to rats treated with Blank-SAP (Figure 6A and B). Here SSP-SAP injected rats spent significantly lower percentage of the test time in the open arms as well as had a significantly lower percentage of entries into open arms ($t_{8,2} = 4.46$, $p = 0.003$ and $t_{8,2} = 5.26$, $p = 0.001$ respectively). The reduction in open arm entries did not appear to be a result of decreased motor activity since no significant reduction in closed arm entries was found ($t_{8,2} = 1.17$, $p = 0.13$). Post mortem tissue analysis revealed SSP-SAP injections, compared to Blank-SAP injections lead to a reduction in NK_{1r}-IR cells (Figure 6C, repeated measures ANOVA (treatment $\times$ region interaction $F_{3,18} = 3.24$, $p = 0.047$). The NK_{1r}-IR cell lesions in the EPM-tested rats were slightly less discrete than observed in the SI-tested rats. Here SSP-SAP injections significantly reduced the number of NK_{1r}-IR cells in the LA, BLA and BLp compared to Blank-SAP injections (Tukey's $q > 3.55$, $p = 0.036$). The number of NK_{1r}-IR cells remaining in the BLA and BLp correlate significantly with the percent of entries into the open arms while a trend towards significance was observed for the number of NK_{1r}-IR cells remaining in the LA and percent of entries into the open arms [Figure 6D–G, (BLA) Pearson's $r = 0.742$, $p = 0.035$ (BLp) $r = 0.843$, $p = 0.0087$ and (LA) Pearson's $r = 0.732$, $p = 0.062$]. Percentage of entries into open arms did not correlate with number of NK_{1r}-IR cells in the

CeA, ($p = 0.312$). These data further support that the NK_{1r}-IR interneurons of the BL and possibly the LA appear to be pivotal in this regulation of anxiety-like behavior as measured by the EPM.

Discussion

As observed in previous studies utilizing this, or similar targeted toxin (Truitt and Coolen, 2002, Levita et al., 2003), SSP-SAP injections ablate approximately 80% of the NK_{1r} expressing cells in an a regionally selective and cell-type specific manner. Specifically, NK_{1r}-IR cells lesions were primarily restricted to the BL without significantly reducing NK_{1r}-IR cells in adjacent CeA as well as not significantly reducing the total number of neurons (NeuN-IR) or even interneurons (GABA-IR). This regional and phenotypic specificity permits valid investigations into the functional role of this population of interneurons within the local circuit of the BL. Rats with ablated NK_{1r} interneurons in the BL displayed anxiety-like responses in the SI test for up to 6 weeks after the injections without showing any trend towards reversal. Also, NK_{1r} cell lesions in the BL/LA also produced anxiety-like responses in the elevated plus-maze similar to that observed in mice (Gadd et al., 2003). These increases in anxiety measures significantly correlated to the number of NK_{1r}-IR cells in the BL, but not in other amygdalar nuclei. Other studies in rats have reported similar regionally specific effects that suggest the BL is critically involved in anxiety and fear related behaviors. Specifically, Sanders and Shekhar demonstrated that GABAA receptor antagonists were effective in inducing anxiety-like behavioral responses when administered into the anterior BL, but not when administered into the CeA (Sanders and Shekhar, 1995b). Interestingly, less specific neuronal lesions of the entire BL (but not CeA) impaired measures of fear and post shock freezing (Koo et al., 2004), as well as conditioned fear in rats when the BL was lesioned prior to conditioning (Koo et al., 2004) or post conditioning (Anglada-Figueroa and Quirk, 2005). This suggests that the BL enhances fear-like responses. Here we are eliminating a small portion of the intrinsic inhibitory neural circuitry of the BL and we increase anxiety-like behaviors. Collectively these studies combined with the current study demonstrate the BL is capable of enhancing fear and anxiety-like responses.

Data presented here is the first to confirm that the vast majority of NK_{1r}-IR cells in the BL are indeed GABAergic interneurons. Thus reductions in GABA concentrations in the BL, by selective lesions of the NK_{1r} interneurons, may induce the anxiety-like behaviors exhibited. GABA in the BL is closely linked to anxiety in rodent models. GABA_A receptor antagonists administered directly into the BL dose dependently increase anxiety-like responses (Sanders and Shekhar, 1991, Sanders and Shekhar, 1995a, Sanders and Shekhar, 1995b, Thielen and Shekhar, 2002), while benzodiazapine antagonists directly into the BL block the anxiolytic properties of systemic benzodiazapine (Sanders and Shekhar, 1995a). However, a general reduction of GABA activity in the BL is unlikely to solely explain the increased anxiety-like behaviors observed following selective NK_{1r} cell lesions. NK_{1r}-IR cells account for only approximately 3% of the BL-GABAergic interneurons and selectively ablating this small population of NK_{1r} cells in the BL did not result in a significant reduction in the overall number of GABA-IR cells in the BL. While such small reductions in GABA of the BL may explain the anxiogenic effect of NK_{1r} cell ablation, it is also likely that the effect is a result of selective loss of the co-expressed neuropeptides. We also report here, for the first-time that NK_{1r} are found on a population of BL interneurons that express CCK, specifically the Type L CCK population of interneurons described by Mascagni and McDonald (2003). We further confirmed that NK_{1r} co-localize with NPY BL-interneurons (Levita et al., 2003), which are part of a larger defined population of somatostatin containing interneurons (McDonald, 1989, McDonald and Pearson, 1989). Somatostatin interneurons of the BL represent an independent population from the CCK interneuronal population (McDonald,

1989, McDonald and Pearson, 1989), thus it would appear that NK_{1r} overlap two distinct populations of BL interneurons (as diagramed in Figure 1B), the CCK type L interneurons, and the somatostatin interneurons (approximately half of which also express NPY). The effects of selective NK_{1r} cell ablation in the BL on the numbers of CCK-IR and SOM-IR interneurons was not investigated in the current study due to lack of sufficient number of BL sections for staining. However, CCK directly placed in the BL of rats has been reported to increase anxiety-like behaviors (Perez de la Mora et al., 2007) and thus it would be unlikely that removing CCK from the BL would result in increased anxiety-like behaviors (although we can not be sure of what influence chronic loss of CCK would have on anxiety-like behaviors). We also did not directly assess the impact of loss of somatostatin in the BL on anxiety-like behavior, but did look at how the NPY division of this interneuronal population was affected by lesions of NK_{1r} cells.

As observed in a similar study (Levita et al., 2003), NK_{1r} interneurons of the BL encompass almost the entire population of local NPY-IR cells and selective ablation of NK_{1r}-IR cells of the BL also resulted in a significant reduction in local NPY-IR cells. This reduction in NPY cells correlated with reduced SI times. NPY agonists are potent anxiolytics and NPY antagonists are anxiogenic when administered to the BL (Kask et al., 2002, Sajdyk et al., 2002a, Sajdyk et al., 2002b). Interestingly, the reduction in NPY-IR cells of the BL did not result in a significant reduction in NPY-IR fiber density within the BL, suggesting NPY fibers in the BL are mainly from sources located outside of the BL. Collectively these data suggest selective ablation of the NK_{1r} interneurons of the BL results in anxiety-like behavior by disruption of a local circuit within the BL and not simply a generic reduction in NPY content.

Is the anxiogenic effect of lesioning NK_{1r} expressing BL interneurons a result of disruption of a specific local circuit?

It is likely that in the intact state, NK_{1r} interneurons in the BL suppress anxiety-like responses from occurring under non-threatening conditions, since removal of these cells increases anxiety-like behaviors. The BL is cortex-like in circuit structure, cell morphology and phenotype, with glutamatergic projection neurons that are regulated by local networks of GABAergic interneurons (McDonald, 2003). These local inhibitory networks likely synchronize the activity of projection neurons by filtering inputs, regulating firing and coordinating global oscillations necessary for appropriate functioning, as described in the cortex (Freund, 2003, Buzsaki et al., 2004, Markram et al., 2004). Activation of BL interneurons generated by glutamatergic afferents of the cortical sensory association area is capable of blunting excitatory postsynaptic potentials in BL pyramidal cells (Rosenkranz and Grace, 1999, Grace and Rosenkranz, 2002, Rosenkranz and Grace, 2003), although the exact phenotype of these interneurons remains unknown. The SOM-GABA interneurons in the BL, which include approximately half of the NK_{1r}-IR interneurons, are reported to form synapses with GABA_A receptors on distal dendrites of the BL projection neurons, and are juxtaposed to excitatory glutamatergic inputs (Muller et al., 2003). These inhibitory inputs are capable of regulating excitatory inputs to the pyramidal cells by blocking the generation of Ca²⁺ dependant postsynaptic potentials in the dendrites and ultimately shunt activation of pyramidal cells (Muller et al., 2003). Additionally, this blockade of Ca²⁺ influx may also reduce synaptic plasticity or spine formation by reducing back propagations within the dendrites (Rainnie et al., 1991b, Buzsaki et al., 2004, Markram et al., 2004, Bacci et al., 2005). NK_{1r} interneurons are ideally placed in the intrinsic neurocircuitry of the BL to modulate excitatory sensory inputs on the distal dendrites of projection neurons. Thus, ablation of these cells may disrupt normal mechanisms of assigning saliency and result in increased anxiety-like responses to non-threatening cues.

What could be the unique role of substance P and NK_{1r} interneurons in the BL in regulating behaviors?

How does loss of such a small number of interneurons in the BL result in such profound behavioral changes? One possible answer is the unique afferent input that regulates these interneurons, i.e., substance P. Substance P input to the BL is known to stimulate GABA interneurons and enhance inhibition of projection neurons via postsynaptic GABA A receptors (Maubach et al., 2001). Also, BL lesions disrupt substance P agonist-induced enhancement of fear conditioning but not substance P-induced motor responses such as foot-tapping (Woolley et al., 2006). However, it has also been reported that suppressing substance P in the amygdala may attenuate some anxiety-like responses. Blocking NK_{1r} in the amygdala blocks stress responses (Boyce et al., 2001, Steinberg et al., 2002), emotional learning (Lenard and Kertes, 2002), decreases activation of the human amygdala by fearful situations (Furmark et al., 2005), and disrupts assigning appropriate salience to both negative (Rupniak et al., 2003) and positive (Gadd et al., 2003) stimuli. There is increasing evidence that coincidental stimulation of distributed inhibitory interneurons is an effective way to synchronize the firing of a population of projection neurons within a nucleus (Bazhenov et al., 2001, Tamas et al., 1998). Thus, substance P release within the BL can selectively activate a widely distributed group of inhibitory interneurons and thus coordinate synchronized firing of the BL projection neurons. More studies are needed to fully understand the seemingly complex role that substance P in the BL may play in regulating anxiety.

Recent anatomical data from our laboratory suggests that the NK_{1r}-IR cells in the BL may be one of the targets of prefrontal cortical inputs (Truitt et al., 2007). The orbitofrontal cortex, in primates, is known to project to and provide critical information related to salience and future reward contingencies to the BL (Schoenbaum G., 2003). A synchronized activation of this pathway is thought to be critical for reward associations that regulate learning and underlie many drug addictions (Schoenbaum et al., 2006). The NK_{1r} interneurons could be one target in the BL to signal salience from the frontal cortex. Such a hypothesis is supported by the findings that substance P is only released into the amygdala following emotionally salient stimuli (Smith et al., 1999, Sergeyev et al., 2005, and Ebner et al., 2004) and that blocking NK_{1r} in the amygdala, while having no effect at resting conditions, nevertheless blocks stress induced changes in behavior (Ebner et al., 2004). Thus, these sparsely distributed NK_{1r} containing interneurons of the BL may have the unique function of synchronizing the firing of BL output with that of prefrontal cortex. While this explanation is speculative, recent electrophysiological evidence demonstrating that both prefrontal cortex stimulation (Rosenkranz and Grace, 2002) and local substance P application (Maubach et al., 2001) induces an inhibitory synaptic potential within the BL projection neurons supports such a mechanism.

In conclusion, we have demonstrated that a small population of GABAergic cells, the NK_{1r} interneurons, in the BL is capable of regulating anxiety-like behaviors. These cells are likely to modulate behaviors by their action within the context of the BL local circuit, and loss of these cells results in exaggerated levels of anxiety-like responses. Furthermore, this study also demonstrates that the targeted toxin approach is a valid tool in the assessment of the functional roles of local circuit classes of interneurons.

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List of Abbreviations

BL	basolateral amygdala
BLa	basolateral amygdala anterior part
BLp	basolateral amygdala posterior part
NK_{1r}	neurokinin1 receptors
NPY	neuropeptide Y
CCK	Cholecystokinin
SAP	saporin
SSP	stable substance P
SP	substance P
IR	immunoreactive
SI	social interaction
EPM	elevated plus maze
LA	lateral amygdala
CeA	central amygdala
SOM	somatostatin
CB	calbindin
PV	parvalbumin
CR	calretinin

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Figure 1.

(A) In the BL, NK_{1r} immunoreactivity is co-localized with GABA- and CCK-immunoreactivity in the interneuron population. Presented here are photomicrographs demonstrating dual immunofluorescence for NK_{1r}- and GABA (top row), NK_{1r} and CCK (middle), and NK_{1r} and NPY (bottom). Examples of single Labeled and dual labeled cells are apparent in the merged column. Scale bar at bottom left represents 50 μ m. Open arrows indicate examples of immunoreactive cells for a given marker while closed arrows represent examples of cells with immunoreactivity to both markers. (B) A schematic diagram of the relative location and abundance of the NK_{1r} interneuron relative to the classes of BL interneurons as defined by Mascagni and McDonald 2003, figure modified with permission.

Figure 2.

Examples of NK_{1R} and NeuN immunolabeling in the targeted BL (i.e., BLa and BLp) and nearby amygdalar structures (i.e., LA and CeA) and lesions of NK_{1R}-IR interneurons. Presented in (A) are examples of NK_{1R} and NeuN staining from adjacent sections (from a blank-SAP rat), as well as the definition of the regions of interest. Sections were taken at approximately -2.5 mm from bregma (Paxinos G, 1986) and overlaid with modified defined regions of interest within the amygdala as per the atlas of Paxinos and Watson (1986). For analyses, the subdivisions of the CeA and LA were combined. Presented in (B) are examples of BL tissue from rats injected with Blank-SAP (top) or SSP-SAP (bottom). Low and high magnification images of NK_{1R}-IR and low magnification image of NeuN-IR from adjacent sections are presented from each rat. Boxes represent the area projected in the center column for each rat respectively. SSP-SAP clearly reduces NK_{1R}-IR in the BL but does not disrupt NeuN-IR, while blank-SAP injections do not appear to affect either type of staining. Scale bars = 50 μ m.

Figure 3.

SSP-SAP injections produced regionally discriminate lesions of NK_{1r} expressing cells. Presented here are means of number of NK_{1r}-IR cells/mm³ in the lateral amygdala (LA), the basolateral amygdala anterior part (BLA), the posterior part (BLP), and the central amygdala (CeA) in Blank-SAP treated rats (n=6) and SSP-SAP treated rats (n=6). (A) Ablation of NK_{1r} expressing cells was mostly localized to the anterior and posterior parts of the BL (i.e., BLA and BLP, respectively) and did not spread to the nearby amygdalar LA or CeA nuclei (with the exception on the LA in 3 rats). A significant reduction in NK_{1r}-IR cells/mm³ between treatment groups was observed only in the BLA and BLP and not in other nuclei (* indicates significance, $p < 0.01$). (B) Additionally, the mean number of NeuN-IR cells/mm³ was counted for each region to determine if any non-selective damage had occurred to any nearby amygdalar nuclei. No significant difference between treatment groups was observed in any of the nuclei. Presented in (C) are the mean volumes used in the calculations for each region of interest by treatment groups. No significant differences in volumes were observed in volumes between treatment groups. Presented in the inset are examples of NK_{1r}-IR fiber labeling (top) in the CeA of Blank-SAP and SSP-SAP rats. No significant reduction in fiber density was observed between groups. The densities of NK_{1r}-IR fibers were determined,

from entire images like those presented in the inset, for each rat using Image J (Rasband, 1997–2005) and means ($\pm$ SEM) for Blank (n=6) and SSP (n=6) were compared (bottom).

Figure 4.

SSP-SAP selectively ablates NK_{1r} expressing cells in the BL. (A) since both the anterior and posterior parts of the BL were affected by toxin injections (figure 3), these regions were combined for phenotypic analysis. The number of NK_{1r}-IR cells/mm³, in the BL, were significantly reduced in rats injected with SSP-SAP (n=6), compared to control rats that were injected with Blank-SAP (n=6, † represents significantly different $p = 0.004$). (B) Despite a significant reduction in NK_{1r}-IR cells/mm³ in the BL by SSP-SAP injections, no significant difference in total neuronal staining was observed [i.e., NeuN-IR was not significantly different between groups ($p = 0.88$)]. Presented in (C) are the number of GABA-IR, NK_{1r}-IR, and co-localized (co) cells/mm³ in tissue from a subset of the Blank-SAP and SSP-SAP rats presented in A (n = 5 each). Both the number of NK_{1r}-IR and

GABA/NK_{1R} co-localized cells/mm³ are significantly reduced in the SSP-SAP rats compared to the Blank-SAP rats (* indicates significantly different, $p = 0.006$). Despite the significant reduction in co-localized cells, no significant difference between treatment groups were observed in the total number of GABA-IR cells/mm³ ($p = 0.93$). Presented in (D) are mean number of NK_{1R}-IR, NPY-IR and co-localized cells/mm³ in tissue from Blank-SAP and SSP-SAP rats. The number of NK_{1R}-IR cells, single labeled NPY-IR cells and co-localized (NPY and NK_{1R}) cell were all significantly reduced in the SSP-SAP rats compared to the Blank-SAP rats († indicates $p < 0.01$, * indicates $p < 0.05$). Presented in (E) are the NPY-IR fiber densities, measured as mean number of pixels above threshold, from the same tissue as D. No significant differences in fiber densities between treatment groups were observed. Presented in (F) are photomicrograph examples of the NPY fiber labeling from a Blank-SAP and an SSP-SAP rat.

Figure 5.

Ablation of NK_{1r} interneurons in the BL reduces social interaction (SI) time [seconds (s) over 5 min test]. Presented in A are the SI times for Blank-SAP and SSP-SAP group of rats (same rats from figures 3–4) prior to treatment (Baseline) and at 1, 3 and 6 weeks following treatment with SAP injections. SI times were significantly reduced in the SSP-SAP rats at all time points after surgery compared to baseline and compared to Blank-SAP rats at each post surgery time point (* indicates significantly different compared to Blank, $p < 0.01$; † indicates significantly different compared to baseline $p < 0.01$). In addition to a treatment effect, SI times significantly correlate only with the number NK_{1r} cells remaining in the BL, but not adjacent nuclei. Presented in (B – E) are scatter plots of week 1 SI times on the abscissa and the number of NK_{1r} cells/mm³ remaining in the BLA (B), BLp (C), LA (D) and CeA (E) on the ordinate.

Figure 6.

Ablation of NK_{1r} cells of the amygdala reduces exploratory behavior in the EPM. In a second experiment the effects of NK_{1r}-IR cell lesions were assessed in the elevated plus maze. Presented here are the mean ($\pm$ SEM) percents of (A) time spent in the open arms and (B) entries into open arms. SSP-SAP (n=5) injected rats spent significantly less time in the open arms than did Blank-SAP (n=5) injected rats. Additionally, the percentage of arm entries into the open arms was significantly reduced in the SSP-SAP injected rat compared to Blank-SAP treated rats. Presented in C are the mean ($\pm$ SEM) number of NK_{1r}-IR cells in the LA, BLA, BLp and CeA of Blank-SAP (open bars) and SSP-SAP (closed bars) treated rats. As observed previously, SSP-SAP injected rats had fewer NK_{1r}-IR cells than did Blank-SAP treated rats in the BL. However, in this experiment, a significant reduction was also observed in NK_{1r}-IR cells in the LA in SSP-SAP treated rats compared to Blank-SAP injected rats. (* indicates significantly different from Blank-SAP, $p < 0.05$). The correlation between percentage of entries into open arms (OA) and NK_{1r}-IR cells remaining in the LA,

BLA, BLp and CeA are presented in D through G, respectively. Here only the NK_{1r} cells in the BL and percentage of entries into open arms had a significant correlation (Pearson's $r = 0.729$, $p = 0.026$; correlation for all other nuclei Pearson's $r = 0.648$, $p = 0.059$).

Table 1

Co-localization of NK_{1r} cells and interneuronal markers in the BL

	NK _{1r}	GABA	NK _{1r}	CCK _L	NK _{1r}	NPY
single-labeled cells	18.4	524	11.6	9.4	14.3	6.8
dual-labeled cells	NK _{1r} + GABA 14.4					
Co-localization	78.3% NK _{1r} + are GABA+ 2.8% of GABA+ are NK _{1r} + 39.7% NK _{1r} + are CCK _L + 41.8% CCK _L + are NK _{1r} + 41.8% NK _{1r} + are NPY+ 88.8% NPY+ are NK _{1r} + NK _{1r} + NPY 6.0					
N	5*					
	4					
	3					

Presented in Table 1 are the mean numbers of single-labeled, dual-labeled or co-localized cells counted bilaterally from 4, 35µm sections through the anterior BL with the number of rats used represented (N) listed on bottom row. Larger CCK_L cells were differentiated from smaller CCK_S cells by diameter of soma, based on criteria established by Mascagni and co-workers (Mascagni and McDonald, 2003), (CCK_L diameter > 10 µm; CCK_S diameter = 10 µm). No NK_{1r} cells co-localized with CCK_S (Data not shown).

* Tissue used in GABA/NK_{1r} co-localization is from Blank-SAP injected rats.