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Regional brain cholecystokinin changes as a function of rough-and-tumble play behavior in adolescent rats

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ABSTRACT

Brain cholecystokinin (CCK) levels have been shown to be elevated in animals defeated during adult social aggression. The present experiment evaluated whether similar effects are evident in prolonged bouts of juvenile social-play fighting, which tend to switch from largely positive to some negative affect after approximately 15 min into a half-hour play session, as indexed by a gradual shift from positively valenced 50 kHz ultrasonic vocalizations (USVs) to negatively valenced 20 kHz USVs. Given the role of CCK in both positive and negative emotional events, we examined levels of CCK-8 in tissue homogenates from 14 brain areas in animals 6 h after a 30 min play bout compared to no-play control animals tested similarly in isolation for 30 min. As with patterns observed following adult defeat, significantly higher CCK levels were evident after play in the posterior neo-cortex compared to no-play control animals (+26%). Levels of CCK were also elevated in the midbrain (+35%). However, unlike in adult aggression, CCK levels were reduced in the hypothalamus (−40%) and basal forebrain (−24%) as compared to no-play animals. Posterior cortex CCK levels were positively correlated to the duration that each animal was pinned ($r = +.50$) which suggests that elevated CCK in the posterior cortex may be related to the negative aspects of play. Hypothalamic CCK levels were negatively related to dorsal contacts and pins (r's = −.57), and suggest that the lower CCK levels may reflect the more positive valenced aspects of play. The data indicate that CCK utilization in the brain is dynamically responsive to rough-and-tumble play.

1. Introduction

Cholecystokinin (CCK) is among the most abundant neuropeptides expressed in the mammalian brain. Sulfated CCK-8 and CCK-4 appear to be the primary endogenous ligands for the CCK system, with sulfated CCK-8 being the primary ligand in the brain [3]. CCK release in the brain has been widely associated with negatively valenced motivational states, such as pain, gastric distension associated with satiety, and anxiety (for summaries, see Refs. [8,14,31]). Also, intravenous administration of CCK fragments increases anxiety in rats [1,7,14,18,25] and humans [12,36]. Similarly, peripheral administration of CCK produces conditioned place aversion in rats [37]. CCK is also a key component of the aggression facilitating

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circuitry in the brain [23], and it is released during inter-male fighting [2]. Recently, we have shown in the resident-intruder male aggression paradigm [5] that defeated intruder male rats exhibit elevated levels of CCK in several regions of the brain which are positively correlated with submissive behavior [31].

In addition to its many aversive motivational/emotional effects, CCK also plays a role in more positively valenced motivational states, such as mating [11,24], drug addiction [9,39] and brain reward processes [10,16,19]. CCK is colocalized with dopamine in ventral striatal dopamine neurons [17], and when CCK is microinjected into the posterior shell of the accumbens, it modulates locomotion, attention and reward, simulating the types of effects typically seen with microinjection of dopamine agonists into the same brain region [19,22].

Thus, it appears that different CCK-based circuitries in the brain can facilitate both negative and positive emotional processes. Accordingly, in the following work, we determined whether the regional brain CCK changes following playful juvenile interactions were similar or dissimilar to brain changes documented following adult aggression. This prominent juvenile behavior has many outward similarities to adult aggression. However, adult aggression is characterized by negative emotional responses (freezing and other indices of defensiveness) while juvenile play is largely a positive incentive, where even ''submissive'' animals vigorously seek continued social engagement [26,34].

In rats, rough-and-tumble play is rewarding for both the dominant and submissive animals of play pairs [26]. Since the motoric vigor of play behavior, with its abundant bodily contact, is outwardly comparable to that seen in adult aggression, the use of these two behavioral models may serve as a way to distinguish brain effects of vigorous social interactions accompanied largely by positive and negative affect, respectively [32]. However, since 50 and 20 kHz ultrasonic vocalizations (USVs)—potential indicators of these positive and negative affective states [21,30]—have not been monitored for extended half-hour testing periods used in our aggression work [20,31], we utilized those measures to monitor fluctuating positive and negative affect during play [21]. If play is characterized largely by positive affect, we anticipated brain CCK changes would be distinct from those resulting from aggression [31]. If play also has negative affective components, some of the resulting brain CCK changes may parallel those seen in adult aggression.

Given the mixed emotional effects of CCK described above and the present discovery that affect as indicated by USVs fluctuated from a more positive to a more negative valence during 30 min rough-and-tumble play sessions, it was reasonable to anticipate that brain areas associated with CCK induced negative affective stimuli would exhibit increased CCK utilization following rough-and-tumble play and may be correlated with the more aversive play behaviors (i.e. duration that the target animal is pinned). Conversely, it was reasonable to expect that in brain areas associated with CCK induced positive affective states would increase CCK utilization following rough-and-tumble play and be positively correlated to the more appetitive dimensions of play activities (i.e. dorsal contacts and 50 kHz USVs that index positive appetitive aspects of play [20,34]).

2. Methods

2.1. Subjects

Animals used in this study were 32 adolescent male Long–Evans rats from eight litters (age 22 days at the start of the study), born and reared in the Bowling Green State University animal facility. Since weaning (at 21 day of age), all test animals had been housed individually in 20 cm × 40 cm × 20 cm translucent polycarbonate cages with corn-cob bedding, with continuous free access to food and water. Temperature was maintained stable at about 22 °C and lighting was on a 12-h light:12-h dark cycle with lights on at 8:00 a.m. All behavioral testing occurred during the light part of the cycle.

2.2. Behavioral testing

At 24 days of age, experimental rats were assigned to be either resident ($n = 8$) or intruder ($n = 8$) play pair partners from the same litters and matched for body weight. Two isolate control animals were also designated per litter with weights matched to the play animals (total $n = 16$). All play testing was conducted in a separate test room, so that the social activities would not disrupt the rest of the colony. Play testing consisted of placing the intruder animal into the homecage of the resident animal for 30 min under dim light (~2 lx). A plastic lid was placed on top of the resident's homecage containing a small hole (6 cm × 6.5 cm) to optimally position the ultrasound sensitive microphone above. Testing was conducted for 2 consecutive days followed by 3–5 days of no testing followed by a final test session (at 28–30 days of age). Control animals did not receive play testing, but were handled and tested in exactly the same way except for the social encounters: Namely, half the controls were placed into the testing room alone in their homecage (own cage controls, $n = 8$) while the other half were placed into the empty cage of a conspecific male (other cage controls, $n = 8$) for a half-hour. Since there were no neurochemical differences in these groups, their data were combined for an overall control condition.

Both the control and play animals were habituated to the test room for 30 min per day on 2 consecutive days before the start of testing. For both experimental and control animals, habituation consisted of placing the individually housed animals in their homecage into the testing room and replacing the cage lid with a translucent plastic lid.

During testing, social interactions of the experimental play animals were recorded with a commercially available video camera and were archived onto DVD, for subsequent scoring of social behaviors. During these recordings, USVs were also monitored using a Pettersson D980 ultrasonic detector (Uppsala, Sweden). Both the frequency division (1/10 division) for 20 kHz USVs and heterodyne channel (tuned to 55 kHz) were recorded onto DVD via separate audio channels.

All behavioral data were analyzed blindly and independently of the biochemical data, and the play behavior was scored blind to the ultrasonic vocalization data. Video tape records were hand scored for two behaviors: (1) dorsal contacts and (2) pinning behavior (see Ref. [34], also see Fig. 15.2 in Ref. [27]). Dorsal contacts consisted of one animal having both its front paws on the dorsal surface of the other

animal; this behavior was scored as frequency of occurrence for each animal. Pinning was scored when the pinned animal was laying in a supine posture with the other animal above the pinned animal. Pinning was scored as frequency of occurrence as well as pin durations. Inter-rater reliability correlations for dorsal contacts, pins and pin durations were $r = .74, .86, .81$, all $P < .05$, respectively. Ultrasonic vocalizations were hand scored sonographically (SASLab Pro, Avisoft Bioacoustics, Germany) with 20 kHz USVs scored from the frequency division channel while the 50 kHz USVs scored from the heterodyne channel.

2.3. *Brain tissue sampling*

Six hours after the final test session, rats were rendered unconscious with ambient carbon dioxide, decapitated and brains as well as pituitary (abbreviation Pit) were extracted. From the extracted brains, the following brain areas (with abbreviations) were dissected: OB, olfactory bulb; Hyp, hypothalamus; FCTX, frontal neo-cortex (consisting of the frontal pole just anterior to the caudate-putamen); CTX1, anterior neo-cortex over basal ganglia; CPU, caudate-putamen; Sept, septal area including some bed nucleus of the stria terminalis and fornix; BF, all basal forebrain ventral to the CPU including the accumbens; Thal, the entire thalamus; Amyg, amygdala and surrounding periamygdala and temporal cortex; CTX2, posterior neo-cortex surrounding the body of the hippocampus; Hipp, body of the hippocampus, excluding dorsal area right above the thalamus; Midbrain, consisting of tectum including colliculi, periaqueductal gray and tegmentum. Gross separation of areas was first achieved by obtaining approximately 2–3 mm coronal sections with a calibrated tissue block, and subsequent fine dissection was conducted on an ice cold platform using microdissection tools. Tissue samples were promptly placed in eppendorf tubes and frozen on dry ice. Samples were stored at −80 °C until assayed.

2.4. *CCK assays*

Biochemical procedures were essentially the same as previously described [4]. The brain samples were homogenized in 0.1N HCl, an aliquot was taken for protein-determination (Bradford Method, Pierce Chemical, Rockford, IL) and another for radio-immunoassays of CCK. The CCK levels were normalized against total protein levels. The utilized method detects CCK-8, CCK-12, CCK-22 and CCK-33, including both sulfated and unsulfated varieties, with a moderate preference for the sulfated form. It has little affinity for CCK-4 or CCK-5, which is scarce in rat brain. The detection limit for CCK is ~2 pg/tube, the ED50 is ~7–10 pg as more fully described in Ref. [4].

3. Data analysis

Ultrasonic vocalizations were analyzed with a two-way repeated measures ANOVA. All other group comparisons were conducted with between subject student's *t*-tests (two-tailed). When correlations are performed, Pearson's correlations were reported, but for comparisons of data not normally distributed, non-parametric Spearman's correlations were employed.

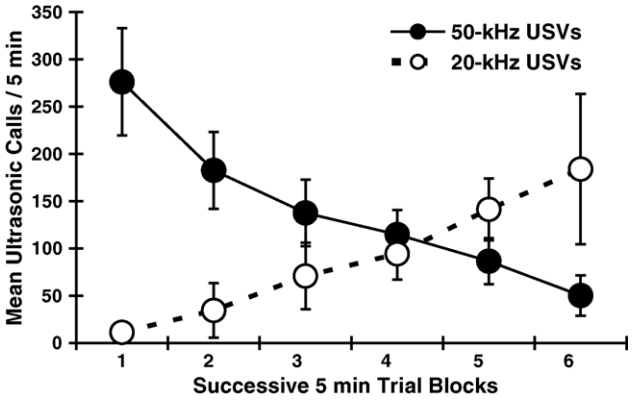

Fig. 1 – Mean ± S.E.M. 50 and 20 kHz ultrasonic vocalizations in successive 5 min trial blocks during a half-hour of rough-and-tumble play testing.

4. Results

4.1. *Behavior*

Across the 30 min play session, levels of 50 kHz USVs gradually decreased, whereas levels of 20 kHz USVs increased, as indicated by a significant vocalization type X trial block interaction ($F(5,14) = 12.63$, $P < .0001$; Fig. 1). Pinning and dorsal contact behaviors did not differ significantly between the resident and intruder animals (all P's $> .05$) and the values by play pair for pins and dorsal contacts are graphed in 5 min successive blocks across the 30 min play session in Fig. 2. The 50 kHz USVs were positively correlated with dorsal contacts, $r = .53$, and pins, $r = .39$ (P's $< .01$, Pearson correlations). The

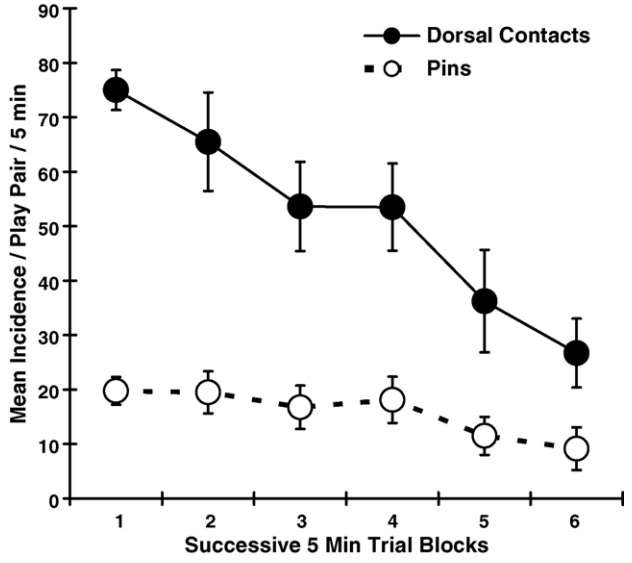

Fig. 2 – Mean ± S.E.M. dorsal contacts and pins for each pair during successive 5 min trial blocks during a half-hour of rough-and-tumble play testing.

20 kHz USVs were significantly negatively correlated with dorsal contacts, $r = -.63$, pins, $r = -.42$ and 50 kHz USVs, $r = -.49$ and positively correlated with average pin durations of the submissive animal, $r = .45$ (P's $< .005$, Spearman correlations).

4.2. *Neurochemical*

CCK levels in all brain regions studied were within the detection limits of the assay [4]. As already noted, CCK levels of control animals that remained in their own homecage during testing did not differ from animals tested alone in a conspecifics homecage in any brain regions analyzed (all P's $> .05$). However, significant CCK changes were evident in the following brain regions of animals allowed to play compared to no-play controls. As summarized in Fig. 3, play animals had increased levels of CCK compared to the isolate controls in CTX2, the posterior cortex ($t(30) = 2.45$, $P < .05$), as well as midbrain samples ($t(30) = 2.45$, $P < .05$); decreased levels of CCK were evident in the hypothalamus ($t(30) = 2.20$, $P < .05$) and the basal forebrain samples that included the nucleus accumbens ($t(30) = 2.62$, $P < .05$). The other brain regions exhibited no significant CCK differences, and the mean ± S.E.M. CCK levels (ng/mg protein) for the control and experimental animals combined were: Pit .173 ± .014; OB .271 ± .018; FCTX 7.472 ± .772; CTX1 6.574 ± .496; CPU .473 ± .024; Sept .404 ± .017; Thal .082 ± .004; Amyg .973 ± .050; Hipp .344 ± .017.

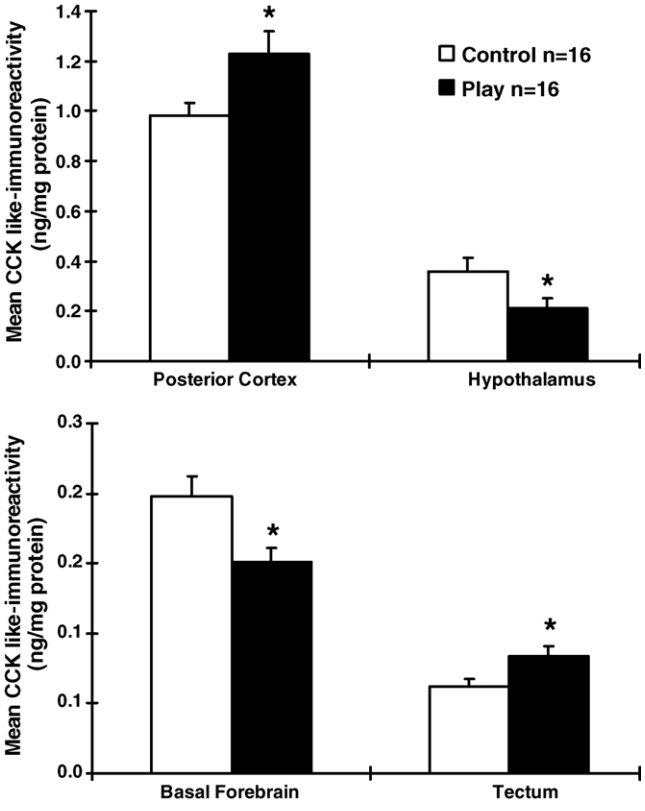

Fig. 3 – Mean ± S.E.M. CCK levels in four brain regions exhibiting significant peptide changes 6 h after rats either received 30 min of rough-and-tumble play as compared to being tested alone. *Note:* Scaling on the Y-axis differs between the top and bottom panels; *P < .05 (between subjects *t*-test, two-tailed).

Dorsal contacts ($r = -.57$, $P < .05$) as well as pins ($r = -.57$, $P < .05$) were negatively correlated with CCK levels in the hypothalamus across all play animals ($n = 16$). CCK levels in the posterior cortex were positively correlated with total duration that each animal was pinned ($r = .50$, $P < .05$) across all play animals ($n = 16$).

5. Discussion

To our knowledge, this is the first study to directly examine CCK or any other neuropeptide change as a result of adolescent rough-and-tumble play behaviors, except for early indirect (subtractive autoradiography) measures of opioid release in the brain [28]. Similar to our recent finding with inter-male aggression [31], we found that a half-hour rough-and-tumble play session elevated CCK levels in the posterior cortex and was correlated with submissive behavior (total pin duration). In the present study, we also found that rough-and-tumble play elevated CCK levels in the midbrain, comparable to the increases in CCK levels in the smaller tegmental samples analyzed in our past inter-male aggression work [31]. Given that CCK elevations in posterior cortex and midbrain observed in this study are similar to those seen in defeated animals in our previous work on adult aggression [31], rough-and-tumble play and adult aggression may share some common neural substrates. However, since no decreases in CCK in hypothalamus and basal forebrain were evident as a function of adult aggressive interactions [31], rough-and-tumble play apparently does affect additional brain CCK systems in ways that are distinct from changes emanating from adult aggression.

Given that the play induced decreases in hypothalamic CCK levels in the present study were correlated with dorsal contacts, which is a positively valenced appetitive play behavior [20,34], it is possible that CCK utilization in the hypothalamus reflects positive motivational aspects of certain social interactions. Decreased levels of CCK in play animals were also evident in the basal forebrain. Given that CCK reductions in the basal forebrain did not correlate with either dorsal contacts or pin duration, we can draw no functional conclusions from this data, but it is possible that CCK in this brain region reflects non-valenced arousal associated with rough-and-tumble play.

The observation that CCK changes in several brain areas following play were different than those observed following aggression affirms that these two social behaviors, although superficially similar, can be empirically distinguished. Such distinctions have also been affirmed by the fact that inter-male aggression and rough-and-tumble play behaviors can be dissociated behaviorally and pharmacologically [33]. Furthermore, behaviorally rough-and-tumble play solicitations are directed at the nape of the neck, while attacks during inter-male aggression are commonly directed at the rump [35].

However, we now document that social play is not consistently positive. Our present analysis of ultrasonic vocalizations (the first half-hour measurement of this type for play) suggests that during prolonged play sessions, positive affect (as indexed by 50 kHz USVs) tends to gradually decrease, and negative affect (as indexed by 20 kHz USVs) gradually increases. Hence, our play tests did not reflect uniform

activation of positive affective systems, as we had originally hoped. Indeed, during the second half of such extended play sessions, behavioral indicators of negative affect emerge which may resemble that seen in adult aggression [31], potentially explaining the adult aggression type neurochemical changes observed in cortex and midbrain.

In anticipation of a dilemma posed by this finding, namely that the vocalization-indexed motivational valence of rough-and-tumble play switches from positive to negative valence after approximately 15 min as indicated by a gradual shift from positively valenced 50 kHz USVs to a preponderance of negatively valenced 20 kHz USVs, we proceeded to evaluate whether rats exhibit place preference for an environment paired with the first 15 min of play to an environment paired with the second 15 min of play. Our preliminary analysis indicates that rats prefer the environment associated with the first half of play [Burgdorf, unpublished data]. This suggests that the second half of the play session is less positive than the first, but it does not indicate that the second half is aversive as compared to a no-play environment. In any event, at present, the gradual shift in motivation during a prolonged play session is inconsistent with the conclusion that one is evaluating a uniformly positive affective social state when half-hour play testing is used.

This leads to a dilemma that cannot be resolved by the present work, namely the extent to which the observed CCK changes were due to the positive versus negative aspects of the social interactions. Considering that posterior cortex and midbrain shifted in the same directions as in defeated animals during adult aggression, we provisionally hypothesize that those two brain changes reflect the negative affective consequences of the playful social interactions. However, we suspect that negative affect engendered during extended rough-and-tumble play is modest compared to that observed during adult social defeat where biting and serious attack commonly occur. Such a conclusion may be supported by the fact that the relative increase in CCK following adult aggression in the posterior cortex was much larger (+68%) than the modest 26% elevation seen in juvenile playing.

Since no regional CCK reductions were observed in adult animals as a function of aggression, perhaps the reductions in CCK levels in the hypothalamus and basal forebrain reflect the positive affective aspects of the juvenile social interactions, especially given the positive correlation between hypothalamic CCK levels and dorsal contacts which reflect positive affective aspects of rough-and-tumble play [20]. This can be directly evaluated in the future by contrasting play pairs that essentially exhibit no 20 kHz USVs indicative of negative affect with play pairs that exhibit higher levels of 20 kHz USVs. A comparable type of evaluation might be achieved through studying the play behavior of selectively bred lines that exhibit high levels of 50 kHz USVs during playful interactions as compared to control line rats [6,29].

Although the present work indicates that some of the CCK regional brain changes that result from playful social interactions are distinct from those evident in adult aggression, both the affective and neurochemical issues deserve more experimental attention. In this context, we note that some past research has also yielded evidence for a bi-valent role of CCK. Both aversive and positive reward processes have been evident in functional CCK studies [13,15,23,25,31,38]. Indeed, different CCK receptor subtypes (CCKA versus CCKB) may mediate positive and negative emotional effects of CCK in different brain regions [1,13,19,40]. More functional work, especially analysis of regional infusions of CCK agonists and antagonists into the implicated brain regions, is needed to disentangle these diverse influences.

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