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POSITIVE EMOTIONAL LEARNING IS REGULATED IN THE MEDIAL PREFRONTAL CORTEX BY GLUN2B-CONTAINING NMDA RECEPTORS

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Abstract—In rats, hedonic ultrasonic vocalizations (USVs) is a validated model of positive affect and is best elicited by rough-and-tumble play. Here we report that modulation of GluN2B-containing NMDA receptors (NMDAR) in the medial prefrontal cortex (MPFC) is involved in positive emotional learning. Rough and tumble play increased both GluN1 and GluN2B NMDAR subunit mRNA and protein levels in the frontal cortex. GLYX-13, a GluN2B-preferring, NMDAR glycine-site partial agonist (1 mg/kg, i.v.) significantly increased positive emotional learning whereas the GluN2B receptor-specific antagonist, ifenprodil (10 mg/kg, i.p.), inhibited positive emotional learning. Animals selectively bred for low rates of hedonic USVs were returned to wild-type levels of positive emotional learning following GLYX-13 treatment. MPFC microinjections of GLYX-13 (0.1–10 μg/side) significantly increased rates of positive emotional learning. Thus GluN2B-containing NMDARs may be involved in positive emotional learning in the MPFC by similar mechanisms as spatial/temporal learning in the hippocampus. **© 2011 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: GLYX-13, GluN2B, medial prefrontal cortex, positive emotion, learning, vocalizations.

Positive affect induces resilience to depression and is an under-examined area for the development of novel therapeutics. Positive affective states, as studied longitudinally in humans, confer resilience to depression and anxiety, and lead to an increase in overall health and a decrease in mortality from all causes (Lyubomirsky et al., 2005). Following a major life stressor, individuals with high positive affect are less likely to develop psychological disorders such as anxiety and depression (Fredrickson et al., 2003). Conversely, individuals who have low levels of positive emotion are at greater risk of developing anxiety disorders, depression, and global health problems (Lyubomirsky et al., 2005). The molecular mechanisms underlying positive affective states are poorly understood and require efficient animal models.

Rat 50-kHz ultrasonic vocalization (hedonic USVs) is a validated model for the study of positive affective states and is best elicited by rough-and-tumble play. Human positive affective states and rat hedonic USVs are elicited by the same stimuli and have homologous neuroanatomical and molecular substrates and alternative non-hedonic interpretations of 50-kHz USVs are not supported by the available data (Burgdorf and Panksepp, 2006; Burgdorf et al., 2008).

GLYX-13 is a tetrapeptide (threonine–proline–proline–threonine) derived from one of the hypervariable regions of the monoclonal antibody, B6B21, and has been shown to act as a partial agonist at the glycine site of the NMDAR (Haring et al., 1991; Thompson et al., 1992; Moskal et al., 2005; Zhang et al., 2008; Burgdorf et al., 2011). GLYX-13 shows preferential modulation of GluN2B containing NMDARs (Zhang et al., 2008). To date, GLYX-13 has been reported to: (1) enhance the magnitude of long-term potentiation of synaptic transmission while reducing long-term depression (Zhang et al., 2008); (2) significantly increase learning in a variety of hippocampus-dependent learning tasks including trace eyeblink conditioning and the Morris water maze in both young adult and learning-impaired aging rats (Burgdorf et al., 2011); (3) markedly reduce delayed (24 h) CA1 pyramidal neuronal cell death produced by bilateral carotid occlusion in Mongolian gerbils when administered up to 5 h post-ischemia (Stanton et al., 2009); and (4) produce a significant antidepressant-like effect in the rat forced swim test (Burgdorf et al., 2010b).

The present study was designed to evaluate the ability of GLYX-13 to facilitate play-induced hedonic 50-kHz USVs, as well as to examine the functional role of GluN2B-containing NMDARs for the generation of hedonic 50-kHz USVs in rats. Further, we evaluated the ability of GLYX-13 microinjections into the medial prefrontal cortex to facilitate positive emotional learning.

EXPERIMENTAL PROCEDURES

Subjects

Rats were purchased from Harlan Sprague Dawley (USA) and housed in Lucite cages with corn cob or sawdust bedding, maintained on a 14:10 light:dark cycle (lights on 8 AM), and given *ad libitum* access to Purina laboratory chow and tap water throughout the study. Three different strains of rats were used in these studies. For the conspecific rough-and-tumble play studies, adolescent (1 month old) Long Evans (LE) rats were used given that

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Abbreviations: CS, conditioned stimulus; LE, Long Evans; MPFC, medial prefrontal cortex; tEBC, trace eyeblink conditioning; US, unconditioned stimulus; USVs, ultrasonic vocalization.

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such young rats exhibit the highest rates of social rough-and-tumble play behavior from among all rat strains tested (Panksepp et al., 1984; Burgdorf et al., 2010a). For analysis of well-controlled heterospecific play (i.e., "tickling" by a human hand, simulating the play of rats, which can bring 50 kHz USVs under 'stimulus-control'), 2–3 month old Sprague–Dawley or F1 Fisher 344 X Brown Norway Rats (FBNF1) were used given that they exhibit especially high rates of adult heterospecific play induced 50-kHz USVs (Burgdorf et al., 2010a). Adolescent heterospecific play was also examined in 1 month old LE rats selectively bred for high or low rates of 50-kHz USVs (Burgdorf et al., 2009). Male 2–3 month old FBNF1 rats were used for the eyeblink conditioning studies given that they are the most widely used strain for such studies (Weiss et al., 1999a,b; Moskal et al., 2005; Burgdorf et al., 2011). All experiments were approved by either Bowling Green State University or Northwestern University Animal Care and Use Committees, and care was taken to minimize the number of animals used and their suffering.

Experiment 1. Effect of rough-and-tumble play on NMDA GluN1 and GluN2B mRNA and protein levels

We examined GluN1, GluN2A and GluN2B NMDA receptor subunit mRNA and protein levels in the frontal cortex (frontal pole anterior to + 2.70 mm from bregma excluding the olfactory bulb) and posterior cortex (dorsal cortex from −0.8 to −5.30 mm from bregma) following conspecific rough-and-tumble play or heterospecific rough-and-tumble play. Six hours after behavioral testing, animals were decapitated and their brains removed for subsequent determination of NMDA receptor mRNA levels by microarray and qRT-PCR and protein levels by Western analysis.

Rough-and-tumble play testing

Conspecific play and heterospecific play were conducted as previously described (Burgdorf et al., 2010a). For conspecific play studies, male or female 32-day old Long Evans rats were assigned to play pairs based on gender and weight matching and were individually placed in the homecage of a conspecific rat for 30 min (experimental group, $n=12$) or alone in a conspecific's homecage (control group, $n=6$). During testing, animals were videotaped and high frequency ultrasonic vocalizations were recorded. Rates of play behavior (dorsal contacts and pins) as well as rates of ultrasonic vocalizations were scored by a blind experimenter with an inter-rater reliability of $r>0.9$. All animals were sacrificed 6 h after testing, the brains rapidly dissected (~90 s), frozen on dry ice and stored at −80 °C until assayed as described in (Burgdorf et al., 2006). Conspecific play animals used for mRNA and protein studies represent the upper quartile of a larger group of animals based on high rates of rough-and-tumble play behavior (pins and dorsal contacts), high rates of hedonic 50-kHz USVs, and low rates of aversive 20-kHz USVs (Burgdorf et al., 2010a). The 6 h time point was chosen given that we had previously reported robust changes in brain mRNA and protein levels following play (Burgdorf and Panksepp, 2006; Burgdorf et al., 2006). To control for social and somatosensory stimulation associated with play, 32 day old LE rats of both genders selectively bred for high rates of hedonic 50-kHz USVs and shown to exhibit a depression resilient phenotype (Burgdorf et al., 2009) were used. Animals received 20 min of experimenter administered rough-and-tumble play stimulation (heterospecific play; experimental group, $n=10$) or 20 min of experimenter administered light touch stimulation (light touch; control group, $n=8$) using trials of 15 s stimulation followed by 15 s of no stimulation across the 20-min test session as described previously (Burgdorf and Panksepp, 2001). Animals were sacrificed 6 h after testing, the brains rapidly dissected (~90 s), frozen on dry ice and stored at −80 °C until assayed.

Trace eyeblink conditioning

Trace eyeblink conditioning (tEBC) was conducted exactly as described in (Burgdorf et al., 2011). Male, 2–3 month old, FBNF1 rats were anesthetized with either a combination of xylazine (13 mg/kg, i.p.; Butler, Dublin, OH, USA) and ketamine (87 mg/kg, i.p.; Akon, Decautor, IL, USA), or isoflurane (to effect) and placed in a stereotaxic device. An incision was made on the top of the skull allowing for retraction of the periosteum. A total of six bilateral holes were drilled into the skull for insertion of stainless steel screws. A strip connector with two Teflon coated stainless steel wires and a non-insulated wire for an animal ground was then placed on the skull. EMG activity was recorded from the orbicularis oculi muscle via the recording wires, which were inserted underneath the skin until they penetrated the upper eyelid of the right eye. A tether holding a connector for relaying EMG activity and a tube for air puff delivery was attached to the strip connector. Dental cement was then placed around the connector and over the screws until the connector was firmly in place. Following surgery, animals were placed on a heating pad and given Buprenex (0.5 mg/kg, s.c.; Reckitt Benckiser, Hull, UK) to alleviate possible discomfort due to surgery. Animals were given a minimum of 5 days to recover before beginning the behavioral training. Rats received injections of 0.9% saline 10 min prior to habituation and each daily training session. All injections were given in a volume of 1 ml/kg. Animals were then subjected to trace or pseudo-conditioning paradigms, as described below. A habituation session preceded testing and was of equal duration to the training sessions. No stimuli were presented during the habituation session. During tEBC, rats received 10 sessions (one session/day) of 30 paired presentations per session of an auditory conditioned stimulus (CS, 250 ms, 8 kHz, 85 dB, 5 ms rise/fall) and, following a 250 ms trace interval, an air puff unconditioned stimulus (US, 100 ms, 4.5 psi [0.31 kg/cm^2] corneal airpuff), with an inter-trial interval (ITI) of 20–40 s (30 s average). Testing for the pseudo conditioning group was identical to the tEBC animals except that the CS and US stimuli were unpaired. Data were collected and analyzed as described previously (Weiss et al., 1999a,b; Burgdorf et al., 2011).

Microarray analysis of gene expression

Microarray and data analysis were conducted as previously described (Kroes et al., 2006; Burgdorf et al., 2010a). Duplicate microarray analyses using frontal cortex (all cortical tissue anterior to +1 mm bregma) and posterior cortex (cortical tissue dorsal to hippocampus posterior to −1 mm bregma) isolated from individual rats 6 h after a 30 min play session ($n=6$ play, $n=6$ controls) were performed in a blind manner. Conspecific play animals were selected for the microarray study based on high rates of play behavior (pins and dorsal contacts), high rates of hedonic 50-kHz USVs and low rates of aversive 20-kHz USVs, with ~25% of all animals tested meeting these criterion. The 6 h time point was chosen given that we have previously reported robust changes in brain mRNA and protein levels following play (Burgdorf et al., 2006, 2010a).

Individual 45-mer oligonucleotides complementary to sequences of 1178 cloned rat CNS mRNAs were synthesized on a PolyPlex™ 96-well oligonucleotide synthesizer (GeneMachines®, USA) and spotted in triplicate onto epoxy coated slides (Telechem, USA) using an OmniGrid™ robotic microarrayer (GeneMachines®). Total RNA was extracted (RNeasy, Qiagen, USA) and used as the substrate for RNA amplification and labeling using the Eberwine protocol (Van Gelder et al., 1990). Two micrograms of Cy5-labeled (experimental) and Cy3-labeled (universal rat reference, Stratagene, USA) amplified RNA (aRNA) were cohybridized on individual arrays at 46 °C for 16 h. Arrays were scanned using two lasers (633 nm and 543 nm) at 5 μm resolution on the ScanArray 4000XL (Packard Biochip Technologies, USA). Raw image files

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