Distinct behavioral phenotypes in novel “fast” kindling-susceptible and “slow” kindling-resistant rat strains selected by stimulation of the hippocampal perforant path

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ABSTRACT

Kindling is a phenomenon of activity-dependent neural circuit plasticity induced by repeated seizures that results in progressive permanent increases in susceptibility to epilepsy. As the permanent structural and functional modifications induced by kindling include a diverse range of molecular, cellular, and functional alterations in neural circuits, it is of interest to determine if genetic background associated with seizure-induced plasticity might also influence plasticity in neural circuitry underlying other behaviors. Outbred Sprague-Dawley (SD) rats were selected and bred for ~15 generations for “fast” or “slow” rates of kindling development in response to stimulation of the perforant path input to the hippocampus. After 7-8 generations of selection and breeding, consistent phenotypes of “fast” and “slow” kindling rates were observed. By the 15th generation “fast” kindling rats referred to as Perforant Path Kindling Susceptible (PPKS) rats demonstrated a kindling rate of 10.7 ± 1.1 afterdischarges (ADs) to the milestone of the first secondary generalized (Class V) seizure, which differed significantly from “slow” kindling Perforant Path Kindling Resistant (PPKR) rats requiring 25.5 ± 2.0 ADs, and outbred SD rats requiring 16.8 ± 2.5 ADs ($p < 0.001$, ANOVA). Seizure-naïve adult PPKS and PPKR rats from offspring of this generation and age-matched adult outbred SD rats were compared in validated behavioral measures including the open field test as a measure of exploratory activity, the Morris water maze as a measure of hippocampal spatial memory, and fear conditioning as a behavioral paradigm of associative fear learning. The PPKS (“fast” kindling) strain with increased susceptibility to seizure-induced plasticity demonstrated statistically significant increases in motor exploratory activity in the open field test and reduced spatial learning the Morris water maze, but demonstrated normal fear conditioned learning comparable to outbred SD rats and the “slow” kindling-resistant PPKR strain. These results confirm that selection and breeding on the basis of responses to repeated pathway activation by stimulation can produce enduring

modification of genetic background influencing behavior. These observations also suggest that genetic background underlying susceptibility or resistance to seizure-induced plasticity in hippocampal circuitry also differentially influences distinct behaviors and learning that depend on circuitry activated by the kindling selection process, and may have implications for associations between epilepsy, comorbid behavioral conditions, and cognition.

KEY WORDS: hippocampus, perforant path, seizures, epilepsy, genetic background, learning, memory, water maze, open field, fear conditioning

ABBREVIATIONS: Sprague-Dawley (SD), Perforant Path Kindling Susceptible (PPKS), Perforant Path Kindling Resistant (PPKR), afterdischarge (AD), afterdischarge threshold (ADT), conditioning stimulus (CS), unconditioned stimulus (US).

INTRODUCTION

Kindling, the phenomenon of seizure-induced neural circuit plasticity, was discovered nearly 50 years ago during the course of experiments investigating the effects of repeated brain stimulation on learning and memory acquisition. Kindling can be induced in species ranging from amphibians to primates by repeated episodes of network synchronization or seizures which cause a progressive, permanent increase in susceptibility to additional seizures (Goddard, et al. 1969). Kindling induces progressive, permanent structural and functional circuit alterations with increasing susceptibility to additional seizures in neural circuits throughout the brain, and eventually results in emergence of recurring spontaneous seizures that define epilepsy (Wada, et al. 1975; Wada and Osawa 1976; Pinel and Rovner 1978; Sayin, et al. 2003, Bertram 2007). The process of kindling has been widely used as an experimental model for investigation of temporal lobe epilepsy, the most common form of poorly controlled human epilepsy (Neligan, et al. 2012). As a phenomenon of activity-dependent neural plasticity and circuit remodeling, kindling can also be regarded as a neurobiological process contributing to progressive features of poorly controlled epilepsy. Because activity-dependent plasticity also underlies processes of learning and memory, it was of interest to determine if genetic background influencing kindling development might also influence other behaviors dependent on neural plasticity.

To pursue these questions, we selected and bred Sprague-Dawley rats for susceptibility or resistance to development of kindled seizures evoked by stimulation of the perforant path input to the hippocampus, a region of the brain implicated in memory formation and epilepsy. In a previous series of studies, rats selectively bred for “fast” or “slow” rates of kindling through the amygdala (McIntyre, et al. 1999; Racine, et al. 1999) demonstrated significant differences in several behavioral measures including more intense reaction to stressors such as fear, decreased

habituation of exploratory behavior on the open field test, and impaired learning in hippocampus-dependent tasks such as the Morris water maze and a delayed alternation task (Mohapel and McIntyre 1998; Anisman, et al. 2000; Anisman and McIntyre 2002; Kelly, et al. 2003; Runke and McIntyre 2008). These behavioral abnormalities were interpreted to suggest that enhanced seizure susceptibility is associated with attentional, emotional, and learning abnormalities potentially implicated in epilepsy and comorbid behavioral conditions (McIntyre and Gilby 2007). To consider the possibility that there might be interesting differences between the plasticity and phenotypes of rats selected on the basis of perforant path versus amygdala stimulation, outbred Sprague Dawley rats selected for “fast” or “slow” rates of kindling development were bred for ~15 generations, and seizure-naïve adult rats from the “fast” and “slow” strains were examined for behavioral phenotypic differences in validated behavioral measures including the open field test (Prut and Belzung 2003), the Morris water maze (Morris, et al. 1982; Morris 1984), and fear conditioning (Tronson, et al. 2012). The “fast” kindling and “slow” kindling strains selected in response to stimulation of the perforant path input to the hippocampus exhibited statistically significant differences in these behavioral measures, suggesting that genetic background influencing seizure-induced plasticity also influences functional properties of neural circuitry underlying fear conditioning, motor activity, and spatial learning. Preliminary results of these experiments have been published in abstract form (Langberg 2012).

MATERIALS AND METHODS

Surgical procedures

Outbred Sprague-Dawley (SD) male and female rats (Harlan, Inc.) were anesthetized with isoflurane (1-5% for induction, 1-2% for maintenance) and implanted through a craniotomy with a bipolar stimulating and recording electrode in the angular bundle of the perforant path (8.1mm posterior, 4.4mm lateral, 3.5mm ventral to bregma). Bupivacaine (0.125%) was injected for analgesia around the incision and operative site prior to the procedure, and lidocaine was applied at stereotaxic pressure points. The electrode was fixed to the skull with acrylic. Flunixin 2 mg/kg IM was administered following the procedure for postoperative analgesia. All procedures were approved by the University of Wisconsin Institutional Animal Care and Use Committee and were in conformity with institutional and national guidelines.

Kindling procedures

After a 1-2 week recovery period, the afterdischarge threshold (ADT) in response to 1 sec trains of 60 hz biphasic constant current stimulation was determined by application of a graded series of stimulus intensities (100, 200, 300, 400, 500, 700, 900, 1000, 1100, 1300, 1500 μ amp). The ADT was defined as the lowest current intensity that evoked an afterdischarge (AD). On subsequent days, rats received twice-daily (5 days per week) electrical stimulation at the determined ADT to evoke electrographic and behavioral seizures classified according to a modified Racine scale (Racine 1972a; Racine 1972b; Sutula and Steward 1986; Cavazos, Golarai et al. 1991; Cavazos, Das et al. 1994): Class I – arrest of motion or “freezing”, Class II – freezing and automatisms (blinking, facial twitching, drooling), Class III – unilateral extremity clonus, Class IV – bilateral extremity clonus, and Class V – bilateral extremity clonus and loss of postural tone. If a rat experienced 3 ADs at a given stimulus intensity, the intensity for subsequent stimulations was then reduced to the next lowest intensity in the above sequence. This protocol was intended to evoke ADs at the lowest possible (i.e., threshold) intensity.

Stimulation was repeated until 3 Class V seizures were evoked. The kindling rate was defined as the number of ADs required to evoke the first secondary generalized (Class V) seizure.

Breeding

Using the above procedures, outbred SD rats were selected for “fast” or “slow” rates of kindling development in response to stimulation of the perforant path, which is the major afferent input to the hippocampus. SD rats typically develop the first Class V seizure after ~14-16 ADs using the above protocol of perforant path stimulation (Sutula and Steward 1986; Cavazos, et al. 1991; Cavazos, et al. 1994). Rats experiencing the first Class V seizure with ≤ 10 ADs were regarded as “fast” or kindling-susceptible. Rats that experienced the first Class V seizure after ≥ 20 ADs were regarded as “slow” or kindling-resistant. Pairs of male and female “fast” (<10 ADs) or “slow” (>20 ADs) rates of kindling to the first Class V seizure were mated. Offspring were implanted with a perforant path electrode after maturation into adulthood, and received kindling stimulation according to the above protocol. Progeny with “fast” or “slow” rates of kindling in response to perforant path stimulation based on the above criteria were then bred in successive generations to select for the phenotype of “fast” perforant path kindling susceptibility or “slow” perforant path kindling resistance.

Behavioral testing

After ~ 15 generations, approximately equal numbers of adult male and female “fast” kindling, “slow” kindling, and outbred SD control rats which did not undergo kindling procedures were compared in validated behavioral measures including the open field test (Pruet and Belzung 2003), the Morris water maze (Morris, et al. 1982; Morris 1984), and fear conditioning (Tronson, et al. 2012).

Open field: Exploratory motor activity was assessed on 4 consecutive days in a standardized open field consisting of a 5x5-foot tiled floor area divided into 25 equal-sized squares enclosed by a 3-foot-high opaque, plexiglass wall. Each trial session was initiated by placing the rat into the field facing an inside corner of the enclosed area. Movement was tracked manually for one 5-minute trial on each of 4 consecutive days. The number of lines crossed, defined by both forepaws crossing a line, was recorded in each trial.

Morris water maze: A circular tank (117-cm diameter) containing water ($26 \pm 1^{\circ}\text{C}$) opacified by mixing with evaporated milk was filled to a depth of 25 cm. Four points on the perimeter of the pool were designated north (N), east (E), south (S) and west (W). An 8 x 8 cm plexiglass platform was positioned in the center of one quadrant, 1 cm below the water surface. On training day 1, each rat was placed in the pool for 60 sec of swimming habituation without the platform present. On trial days 2-5, the rats experienced 6 swimming trials per day. The quadrant in which the platform was located remained constant, but the point of immersion into the pool changed from trial-to-trial in a predetermined, systematic manner. The latency from immersion into the pool to escape onto the platform was recorded for each trial. Upon mounting the platform, the rat was given a 30-sec rest before the start of the next trial. Four hours after the final trial on day 5, a probe trial was performed with the platform removed from the pool to assess spatial preference as a measure of memory. For this probe trial, the rat was placed back into the pool in the quadrant opposite the previous platform location, and the swim path and the number of virtual crossings over the previous platform location were recorded during 60 sec of free swimming.

Fear conditioning: Fear conditioning was assessed in a square, opaque black plexiglass chamber (10 x 10 x 5 inches) with a floor of 5 inch diameter metal conducting bars (San Diego

Instruments, San Diego, CA). Movement in the chamber was detected by interruption of a 16 x 16 array of perpendicular infrared beams recorded by photoelectric sensors within the chamber. Motor inactivity or “freezing” was measured as the total time the animal was motionless during 30 sec recording intervals. Motor activity was examined during periods of baseline training, fear conditioning in response to foot shock associated with an auditory cue, and context/auditory cue recall. The duration of “freezing” or motor inactivity during fear conditioning and cue/context recall periods was compared to duration of “freezing” during comparable intervals in the baseline period.

Training and fear conditioning: During the training period the rats were housed individually for two days, and then were transported for 3 consecutive days in covered, single cages into a dimly illuminated behavioral testing room where each rat was accommodated to the testing environment by a period of quiet handling for 5 minutes. On day 1 of fear conditioning, the rats were acclimated for 1 hour in a single cage adjacent to the testing room, and were then placed into the fear conditioning chamber. Each rat was allowed to explore the chamber for 2 minutes, and was then exposed to a 70 dB frequency tone for 30 seconds (the conditioning stimulus or CS). During the last two seconds of the CS, the animals received a ~1.0 mA sinusoidal shock (the unconditioned stimulus or US) delivered to the paws by the conducting floor of the chamber. After a 2 minute recovery period with no activation of the US or CS, another identical sequence of a 30 second CS and a ~2 second shock of ~1.0 mA (US) was applied. After a 1 minute recovery period, the rats were removed from the conditioning chamber, which was cleaned with 70% ethanol after each use. Throughout the training and testing procedures, the rats were kept in different waiting rooms to reduce cues in the physical environment that might evoke anticipation of the CS or US.

Context Testing Context recall in the spatial environment of the testing chamber was assessed at 24 hours after training and fear conditioning. Rats were similarly transported to another distinct waiting room for 30 minutes prior to context testing. Each rat was then placed for 6 minutes into a corner of the same training chamber without auditory cue (CS) or foot shock (US). Exploratory behavior and motor inactivity or “freezing” were recorded. After completion of this context testing period, each rat was again similarly transported to another remote waiting room and the chamber was again cleaned with 70% ethanol.

Auditory Cue Recall Testing At 1 hour after completion of context testing, each rat was again transported and returned to the training/testing chamber modified by insertion of a triangular insert box containing a vanilla extract soaked paper towel to reduce olfactory and spatial cues from previous sessions. The auditory cue testing protocol was identical to the fear conditioning protocol except for absence of the 2 sec foot shock (US), and consisted of the following sequence: 120 sec of exploration, 30 sec of the 70 dB auditory cue (CS), 120 sec of exploration, and another 30 sec of the 70 dB auditory cue (CS) followed by 60 sec of exploration. The cue testing chamber was then cleaned with 70% ethanol prior to testing additional rats.

Statistical methods

Kindling rates and behavioral measures were expressed as the mean +/- standard error of the mean (S.E.M.). Differences across multiple groups were assessed for statistical significance by analysis of variance (ANOVA) with post- hoc individual comparisons as appropriate (Dunn’s correction, Holm-Sidak). When data were not normally distributed, nonparametric tests were performed. Differences were regarded as significant at confidence levels of $p < 0.05$.

RESULTS

Selection of “fast” kindling-susceptible and “slow” kindling-resistant rat strains

Using the kindling, selection, and breeding protocols described in *Materials and Methods*, after ~ 7-8 generations, stable “fast” or “slow” rates of kindling were reliably observed compared to outbred SD controls. Selection and breeding to the 15th generation produced distinct phenotypes characterized by “fast” rates of kindling to criterion of the first Class V seizure (10.7 ± 1.1 ADs, $n = 13$) or “slow” rates to the first Class V seizure (25.5 ± 2.0 ADs, $n = 10$) compared to outbred SD controls (16.8 ± 2.5 ADs, $n = 12$) (see Fig. 1). The differences across the groups were significant ($F = 17.423$, $df = 2$, $p < 0.0001$, one way ANOVA). Rats characterized by “fast” rates to criterion were referred to as “fast” Perforant Path Kindling Susceptible (PPKS) rats, and rats characterized by “slow” rates to criterion were referred to as “slow” Perforant Path Kindling Resistant (PPKR) rats. The difference in kindling rate between “fast” PPKS and “slow” PPKR rats (10.7 ± 1.1 ADs vs. 25.5 ± 2.0 ADs) was also significant ($p < 0.001$, post-hoc pairwise comparison, Holm-Sidak method). Both strains also differed from SD controls (PPKS, $p = 0.00125$; PPKR, $p = 0.00125$; Holm-Sidak post-hoc pairwise comparisons). There were no significant differences in mean AD threshold ($p = 0.707$) or mean AD duration ($p = 0.439$) for the first evoked AD among the PPKS, PPKR, and SD rats. Gender related differences in kindling rates, AD threshold, and AD duration were not observed.

Behavioral differences in PPKS, PPKR, and outbred SD rats

Open Field Activity

Exploratory motor activity as assessed in a standardized open field demonstrated consistent differences among equal numbers of male and female adult seizure-naïve PPKS ($n =$

10), PPKR (n = 10), and outbred SD rats (n = 10). On the initial day of 4 consecutive days of open field testing, there were significant differences in the outcome measure of mean number of lines crossed during a 5 minute testing interval among PPKS, PPKR, and outbred SD rats ($F = 6.188$, $df = 2$, $p = 0.006$, one-way ANOVA, Fig. 2A). The difference between mean number of lines crossed by PPKS rats and PPKR rats was also significant ($p = 0.002$, pairwise post-hoc comparison, Holm-Sidak). This strain-dependent pattern of differences in open field performance was also observed on day 2 of testing and diminished on days 3 and 4 of testing, but overall during the 4 days of open field testing there were significant effects of strain ($F = 9.963$, $df = 2$, $p < 0.001$, 2-way ANOVA) and trial day ($F = 3.049$, $df = 3$, $p = 0.032$, 2-way ANOVA) (Fig. 2B). There was no significant interaction of strain and trial day.

Morris Water Maze

There were significant differences between adult seizure-naïve PPKS (n = 7; 2 female, 5 male), PPKR (n = 5; 4 female, 1 male), and SD (n = 6; all male) rats in latency to escape onto the fixed, hidden platform in the Morris water maze over the course of the trial days that varied as a function of strain and day of the trial. Swimming speeds did not differ among the strains, and within the PPKS and PPKR groups there were no gender differences. Across the 4 trial days, there were significant differences that varied by strain ($F = 8.651$, $df = 2$, $p < 0.001$, 2-way ANOVA,) and trial day ($F = 9.471$, $df = 3$, $p < 0.001$, 2-way ANOVA). The differences as a function of strain were greater than would be expected by chance after allowing for effects of differences in trial day, but there was no strain x day interaction ($p = 0.2$). There was a strain difference observed between PPKS and SD rats across all trial days ($F = 14.345$, $p < 0.001$), but not between PPKR and SD rats ($F = 2.033$, $p = 0.155$).

On the initial trial day, the latency to find the hidden platform was greater in both PPKS and PPKR rats compared to SD controls (Fig. 3A, $p = 0.009$, Kruskal-Wallis ANOVA on ranks). Pairwise post hoc comparison of PPKS and PPKR to the SD rats on trial day 1 was also significant (both $p < 0.05$, Dunn's correction). Although the performance of all strains improved over trial days 1-4, indicating that learning occurred in all strains, there were differences in the escape latency to the platform on trial day 4 between the PPKS and PPKR rats compared to the SD rats ($H = 17.408$, $df = 2$, $p < 0.001$, Kruskal-Wallis 1-way ANOVA on ranks). Post-hoc analysis revealed that on day 4 the latency to platform was greater in PPKS rats compared to SD rats (68.2 ± 13.7 vs. 41.2 ± 5.9 , $p < 0.05$, Dunn's correction) and PPKR latency to platform on day 4 was also greater than SD rats (47.7 ± 4.3 vs. 41.2 ± 5.9 , $p < 0.05$, Dunn's correction). There was no significant difference between PPKS and PPKR strains on day 4 ($p = 0.51$). The strain differences between both PPKS and PPKR vs. SD rats on the final day of trials are small compared to the overall robust effects across both strain and day, but indicate that strain effects are still detectable even after robust water maze learning has occurred.

Confirming that rats in all groups learned the location of the platform during trial days 1-4, the probe test performed with the platform removed at 4 hours after the completion of the last trial on day 4 demonstrated that rats from each strain spent a comparable percentage of time swimming in the quadrant that had been the location of the platform during 60 sec of free swimming (Fig. 3B).

Fear Conditioning

On day 1 of fear conditioning, equal numbers of male and female adult seizure-naïve rats from each strain were placed into the fear conditioning chamber, and were allowed to explore the

chamber for a baseline period of 2 minutes prior to a 30 sec period of auditory cue (CS) accompanied by foot shock (US) during the last 2 sec of the cue interval. There were significant differences in freezing or motor inactivity in the chamber during this 120 sec baseline interval in seizure-naïve PPKS rats ($n = 20$), PPKR rats ($n = 20$), and SD control rats ($n=20$) that varied as function of strain ($F = 6.724$, $df = 2$, $p = 0.002$, 1-way ANOVA, Fig. 4A). Post-hoc comparisons revealed that PPKR rats demonstrated more freezing during the baseline interval than PPKS rats (28.3 ± 2.03 sec vs. 21.0 ± 2.3 sec, $p = 0.022$, Holm-Sidak) and also more freezing compared to SD controls (28.3 ± 2.03 sec vs. 18.3 ± 1.6 sec, $p = 0.017$, Holm-Sidak). There were no gender related differences.

At 24 hours after undergoing fear conditioning with a sequence of 2 pairs of CS-US stimuli as described in the methods, context recall was assessed in the same chamber by measuring freezing during a total interval of 360 seconds. All rats demonstrated robust fear context learning as demonstrated by statistically significant increases in freezing during the first 120 sec interval compared to the baseline training period of 120 sec ($F = 25.809$, $df = 2$, $p < 0.001$, 2-way ANOVA, Fig. 4B). There was no effect of strain ($F = 2.28$, $p = 0.107$). The ~ 2-fold increases in freezing during 120 sec of context testing compared to baseline for PPKS, SD, and PPKR were all significant (post-hoc pairwise comparisons, $p < 0.001$, Holm-Sidak).

There were also differences in freezing during the entire 360 sec context recall testing period among PPKS, PPKR, and SD rats that varied as function of strain ($F = 3.294$, $p = 0.045$, 1-way ANOVA, Fig. 4C). Freezing behavior during the total 360 sec period of context recall was also greater in PPKR rats than SD controls (175.3 ± 13.6 sec vs. 122.8 ± 15.1 sec, $p = 0.013$, Holm-Sidak). Overall, there was a pattern of increased freezing in PPKR vs. SD and PPKS rats during both the 120 sec baseline measurement period prior to fear conditioning and during the

360 sec period of context recall testing at 24 hours after fear conditioning (compare Fig. 4A vs. 4C).

At 1 hour after completion of context recall testing, auditory cue recall was assessed in the chamber modified by insertion of a triangular insert box containing a vanilla extract soaked paper towel to reduce olfactory and spatial cues from previous sessions. Fear conditioning cue learning was confirmed by ~ 2-fold increases in freezing during a 120 sec period following a 30 sec auditory cue (CS) without foot shock compared to the baseline 120 sec interval in PPKS, SD, and PPKR rats. All rats demonstrated robust auditory cue conditioned fear learning as demonstrated by statistically significant increases in freezing during the first 120 sec interval compared to the baseline training period of 120 sec ($F = 57.664$, $df = 1$, $p < 0.001$, 2-way ANOVA, Fig. 4D). There was also an effect of strain ($F = 16.693$, $df = 2$, $p < 0.001$).

DISCUSSION

In this study, selection and breeding across 15 generations for “fast” and “slow” rates of kindling development to the milestone of the first Class V seizure in response to stimulation of the perforant path in SD background rats resulted in stable, reproducible, distinct strains with phenotypes of kindling susceptibility or resistance which we refer to as PPKS and PPKR rats, respectively. The behavioral analysis of PPKS and PPKR rats demonstrated that the strains differed not only in susceptibility or resistance to kindling plasticity, but also in specific behaviors that depend in part on circuitry activated during the kindling selection process. The “fast” kindling PPKS strain, selected by stimulation of the perforant path input to the hippocampus, demonstrated statistically significant increases in motor exploratory activity in the open field test and reduced spatial learning in the Morris water maze, but exhibited normal fear

conditioning learning comparable to the “slow” kindling-resistant PPKR strain and outbred SD rats.

Strain-dependent differences in behaviors in PPKS, PPKR, and SD rats are independent of seizures

The strain dependent differences in behaviors were observed in 15th generation adult PPKS and PPKR rats that had not undergone any electrode implantation, invasive recording, or perforant path stimulation. While we did not systematically monitor these rats for spontaneous seizures or EEG abnormalities, random observation during handling and routine animal husbandry did not reveal spontaneous seizures. As rats kindled by repeated perforant path stimulation typically do not exhibit spontaneous seizures until after ~ 100 seizures have been evoked (Sayin, et al. 2003), it is likely that the observed behavioral differences can be interpreted as strain dependent behavioral phenotypes rather than consequences of recent evoked or ongoing spontaneous seizures. These observations imply that the functional behavioral differences between the strains are potentially a consequence of repeated seizures and breeding in preceding generations rather than recent seizures.

PPKS rats demonstrated increased open field exploratory activity compared to PPKR and SD rats

Open field measures revealed a ~ 2-fold increase in exploratory motor behavior in kindling-susceptible PPKS rats compared to kindling-resistant PPKR rats, with the background SD strain demonstrating exploratory motor activity that was intermediate between the PPKS and PPKR strains. Exploratory motor activity measured by the number of field lines crossed varied inversely to the number of ADs characterizing kindling-susceptibility or kindling-resistance across strains, ie., “fast” kindling PPKS rats selected on the basis of fewer ADs to the Class V

milestone demonstrated greater numbers of field line crossings. Conversely, in the PPKR strain, selected on the criteria of greater numbers of ADs to the milestone, fewer line crossings were observed.

Increased open field activity has been variably interpreted as an indication of behavioral capabilities for adaptive exploration and is also been regarded as a measure of anxiety (Prut and Belzung 2003). Additional investigation would be required to further characterize increased open field activity in kindling-susceptible PPKS rats as a manifestation of anxiety or an adaptive exploratory response. While the inverse relationship between kindling susceptibility and open field motor activity is noteworthy, how genetic background differences in the strains influencing seizure-induced plasticity might also influence neural mechanisms required for exploratory motor activity or anxiety is entirely speculative. Although the basis for the differences in open field motor activity is uncertain, reduced motor activity was observed in the “fast” kindling-susceptible PPKS strain by the 3rd day of testing suggesting that some form of learning or adaptation was occurring in this strain.

Increased exploratory motor activity in the PPKS strain was also observed during the baseline phase of fear conditioning. Whether this observed increase in motor activity is related to the open field measures is uncertain, but indicates that increased spontaneous motor activity is observed in the kindling-susceptible PPKS strain in different environmental circumstances.

PPKS rats demonstrate reduced spatial learning in the Morris water maze compared to PPKR and SD rats

While PPKS, PPKR, and SD rats all demonstrated spatial learning as manifested by shorter escape latencies during the 4 days of testing in the Morris water maze, there were robust strain differences. The kindling-susceptible “fast” PPKS rats required more swimming time to

locate the hidden platform than the kindling-resistant “slow” PPKR and SD rats across days 2-4 of testing. The trajectory of reduced escape latencies across days 2-4 of the trial and the spatial probe test outcomes comparable across strains demonstrates that spatial learning did occur in PPKS, PPKR, and SD rats. There were differences among the strains, however, consisting of significantly longer escape latencies in PPKS compared to PPKR and SD rats during days 2-4, as well as small but significantly longer escape latencies on day 4 in PPKS rats. As swimming speeds were comparable across strains, these observations suggest that spatial learning may be diminished in PPKS compared to the PPKR and SD rats.

Deficits in performance in the Morris water maze have been regarded as indications of hippocampal dysfunction underlying spatial memory acquisition and retention (Morris, et al. 1982; Morris 1984; Hannesson, et al. 2001; Hannesson, et al. 2004; Sayin, et al. 2004). The observed differences in PPKS vs. PPKR and outbred SD rats implicating an underlying hippocampal abnormality is potentially of interest as the PPKS strain was selected on the basis of rapid kindling rates in response to repeated stimulation of the perforant path, the major input to the hippocampus. These functional observations suggest the possibility that hippocampal circuitry differences between PPKS and PPKR/SD rats contributing to vulnerability to seizures may also functionally impair other hippocampus-dependent behaviors. It is also of potential interest that while each strain demonstrated spatial learning, the latency to find the hidden platform was greater in both PPKS and PPKR rats compared to SD controls not only on day 1 but also on day 4. The latter result suggests that genetic background influencing susceptibility or resistance to seizure-induced plasticity in both strains may also have subtle behavioral effects relative to more normal outbred control strains.

Fear conditioning learning is comparable in PPKS, PPKR, and SD rats

In contrast to measures of open field activity and performance in the Morris water maze, fear conditioning learning was comparable among PPKS, PPKR, and SD rats. While measures of spatial fear context memory and auditory cue fear recall were comparable across the strains, there were subtle motor activity differences consisting of increased motor “freezing” in PPKR rats during baseline observation and during spatial fear context recall which reached statistical significance compared to SD rats and also appeared as a trend relative to PPKS rats. The possible relationship of increased motor “freezing” in PPKR rats, which can also be characterized as reduced motor activity in these fear conditioning measures, to the reduced open field motor activity in PPKR rats is uncertain. It is clear, however, that each of the strains demonstrated robust fear context memory and auditory cue fear recall.

Comparisons of “fast” kindling PPKS and “slow” kindling PPKR rats to “fast” kindling and “slow” kindling rats selected by amygdala stimulation

The generation of distinct “fast” and “slow” kindling strains in response to stimulation of the perforant path confirms previous studies demonstrating that stable kindling susceptible or resistant phenotypes can be produced in response to breeding and selection in response to stimulation of the amygdala (McIntyre, et al. 1999; Racine, et al. 1999). Taken together, these studies demonstrate that strains of kindling-susceptible or kindling-resistant rats can be developed in response to selection pressures of stimuli applied to different pathways. As repeated seizures evoked by kindling induce behavioral and cognitive comorbidities across a variety of domains (Sutula, et al. 1995; Adamec and Young 2000; Klaynchuk 2000; Kotloski, et al. 2002; Botterill, et al. 2014), it is of interest to consider whether seizure-naïve rats from “fast” or “slow” kindling strains selected by stimulation of different pathways might demonstrate behavioral differences that depend on selection pathway. Seizure-naïve “fast” rats from strains

selected by both perforant path and amygdala stimulation demonstrated apparently comparable patterns of increased open field activity, and also deficits in the Morris water maze consistent with spatial memory impairments. Variants of the water maze incorporating cued and uncued tasks in the amygdala-selected Long-Evans derived strains also suggested a component of attentional disturbance (Anisman and McIntyre 2002), but these additional tasks have not yet been assessed in the perforant path-selected SD-derived strains.

Given that the circuitry of the amygdala is implicated in fear learning and that amygdala kindling impairs auditory fear conditioning learning in outbred Long-Evans rats (Botterill et al. 2014), it is of interest that differences in fear conditioning measures were not apparent in seizure-naïve rats from the perforant path selected strains examined in this study. These differences suggest that selection pressure applied to specific neural pathways and systems during the kindling process may result in distinct behavioral phenotypes in tasks that depend on the activation of circuitry including the kindled pathway, as noted previously (Hannesson, Howland et al. 2005; Hannesson, Pollock et al. 2008). Direct comparison of the “fast” kindling-susceptible or “slow” kindling-resistant strains selected in response to stimulation of the perforant path vs. amygdala at behavioral, cellular, and molecular levels would be of considerable neurobiological interest. However, it is worth noting that the strains of “fast” and “slow” kindling rats selected in response to amygdala stimulation were derived from a background first generation cross of a Long-Evans hooded and Wistar rats, while the PPKS and PPKR strains were derived from SD background.

Implications for potential relationships of genetic background to seizure-induced circuit plasticity and vulnerability to brain injury, epilepsy, and comorbidities

Breeding and selection for behavioral and phenotypic traits is a well-established process in animal husbandry and farming, and this study has confirmed that direct brain stimulation can also be used to select for phenotypic traits including capacity for activity-dependent neuronal and circuit plasticity and associated distinct behaviors. The approach pursued with this experimental design was to select rats for breeding based on “fast” or “slow” rates of kindling, which is a biological phenomena of seizure-induced circuit plasticity. The studies demonstrated that reliable, enduring susceptibility or resistance to seizure-induced plasticity evoked by kindling can be produced over 7-15 generations, and that the susceptibility or resistance to seizure-induced circuit plasticity is associated with distinct behaviors. The current experiments were not sufficient to determine whether other forms of activity-dependent plasticity such as synaptic plasticity (eg., long-term potentiation or depression) or lesion-induced circuit remodeling also vary systematically as a function of strain. In regard to the latter possibility, however, preliminary studies have revealed structural (Hutchinson, Rutecki, et al. 2012), molecular (Langberg, Sutula 2013), and other functional differences between PPKS, PPKR, and SD rats that are pertinent to vulnerability to brain injury (Hutchinson, Rutecki et al. 2010), as well as development of post-traumatic epilepsy (Cech, Hanson, et al. 2012) and comorbidities of traumatic brain injury (Rutecki, Langberg, et al. 2013). These preliminary observations and the current results suggest that “fast” kindling-susceptible and “slow” kindling-resistant rats may serve as valuable neurobiological tools for multidisciplinary efforts to characterize relationships between genetic background influencing learning, memory, and a variety of brain disorders. We have broadly referred to underlying biological differences between the strains as “genetic background”, but additional investigation of molecular genetic as well as epigenetic mechanisms

underlying strain differences and possible differences as a consequence of perforant path vs. amygdala stimulation selection pressure would be of considerable neurobiological interest.

CONCLUSIONS

Breeding of Sprague-Dawley rats selected by “fast” and “slow” responses to stimulation of the perforant path during 15 successive generations resulted in distinct strains of kindling-susceptible PPKS (**P**erforant **P**ath **K**indling **S**usceptible) and PPKR (**P**erforant **P**ath **K**indling **R**esistant) rats. Adult seizure-naïve PPKS rats differed from PPKR and SD rats in behavioral measures of increased open field activity and reduced spatial learning the Morris water maze, but demonstrated normal fear conditioned learning comparable to PPKR and SD rats. The novel strains reported in these experiments, as well as similar strains selected on the basis of responses to stimulation of the amygdala, may serve as neurobiological tools for investigation of genetic background influencing learning, memory, and a variety of brain disorders.

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FIGURE LEGENDS

Figure 1 PPKS and PPKR Rats. Outbred Sprague Dawley (SD) rats were selected and bred over successive generations for “fast” or “slow” rates of kindling in response to perforant path stimulation as defined, respectively, by ≤ 10 or ≥ 20 afterdischarges (ADs) to achieve the criterion of the first evoked secondary generalized (Class V) seizure. After ~7-8 generations stable phenotypes with “fast” and “slow” rates of kindling gradually emerged and were referred to as Perforant Path Kindling Susceptible (PPKS) and Perforant Path Kindling Resistant (PPKR) rats. After 15 generations, ~91% of PPKS rats and ~100% of PPKR rats demonstrated kindling rates that were, respectively, faster or slower than the median of controls ($p < 0.001$, ANOVA). Post-hoc comparison demonstrated that PPKS and PPKR strains differed significantly in rate of kindling (#), and both strains differed significantly from SD controls (*).

Figure 2 Kindling-susceptible PPKS rats demonstrate increased open field activity compared to kindling-resistant PPKR rats. (A) Open field activity measured by the number of lines crossed in a 5'x 5' checkerboard area consisting of 25 equal-sized squares was increased in PPKS rats and decreased in PPKR rats compared to SD rats ($p = 0.006$, ANOVA). Differences between the PPKS and PPKR strains were significant ($p = 0.002$, pairwise post-hoc comparison, Holm-Sidak). (B) Strain-dependent patterns of differences in open field performance were also observed on day 2 of testing but diminished on days 3 and 4 of testing. Overall during the 4 days of open field testing there were significant effect of strain ($F = 9.963$, $p < 0.001$, 2-way ANOVA) and trial day ($F = 3.049$, $p = 0.032$, 2-way ANOVA). There were no significant interactions of strain and trial day.

Figure 3 Kindling-susceptible PPKS rats demonstrate impaired acquisition of spatial memory in the Morris water maze compared to kindling-resistant PPKR and SD rats. (A)

There were significant differences in mean latency to locate the hidden platform in the water maze that varied by strain ($F = 8.651$, $p < 0.001$, 2 -way ANOVA,) and trial day ($F = 9.471$, $p < 0.001$, 2 –way ANOVA). The mean latency to find the hidden platform was greater in both PPKS and PPKR rats compared to SD controls on the initial trial day ($p = 0.009$, Holm-Sidak), but each strain demonstrated spatial learning as indicated by reduced latencies on consecutive days of testing. Mean latencies in kindling-susceptible PPKS rats were increased compared to kindling-resistant PPKR and control outbred SD rats on each day of testing, including day 4 ($p < 0.001$, Holm-Sidak) indicating detectable strain effects even after robust water maze learning has occurred. **(B)** The probe test with the platform removed at 4 hours after the completion of the last trial on day 4 demonstrated a comparable percentage of swimming in the quadrant that had been the location of the platform during 60 sec of free swimming confirming that rats in all groups learned the location of the platform during trial days 1-4.

Figure 4 Fear conditioning in kindling-susceptible PPKS, kindling-resistant PPKR, and SD rats. (A) PPKR rats demonstrated more freezing or motor inactivity during the 120 sec baseline testing than PPKS or SD rats ($p = 0.002$, ANOVA). **(B)** All rats demonstrated robust fear context learning as demonstrated by statistically significant increases in freezing during the first 120 sec interval compared to the baseline training period of 120 sec ($F = 25.809$, $p < 0.001$, 2 –way ANOVA). All pairwise differences between baseline and context were significant ($p < 0.001$). **(C)** Differences in total freezing during the 360 context recall testing period were also observed among PPKS, PPKR, and SD rats that varied as function of strain ($p = 0.045$, ANOVA)

Freezing behavior during the total 360 sec period of context recall was also greater in PPKR rats than SD controls (175.3 ± 13.6 sec vs. 122.8 ± 15.1 sec, $p = 0.004$, t-test). **(D)** Fear conditioning cue learning was confirmed by the ~ 2-fold increases in freezing during a 120 sec period following a 30 sec CS without foot shock compared to the baseline 120 sec interval in PPKS, SD, and PPKR rats. Robust auditory cue fear learning was demonstrated by statistically significant increases in freezing during the first 120 sec interval after delivery of an auditory cue conditioning stimulus (CS) compared to the baseline training period of 120 sec ($F = 57.664$, $p < 0.001$, 2 –way ANOVA). There was also an effect of strain ($F = 16.693$, $p < 0.001$).

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