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Responsiveness of rat fetuses to sibling motor activity: Communication in utero?

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Abstract

Previous research has revealed that fetuses detect and respond to extrauterine stimuli such as maternal movement and speech, but little attention has been cast on how fetuses may directly influence and respond to each other in the womb. This study investigated whether motor activity of E20 rat fetuses influenced the behavior of siblings in utero. Three experiments showed that; (a) contiguous siblings expressed a higher frequency of synchronized movement than noncontiguous siblings; (b) fetuses that lay between two siblings immobilized with curare showed less movement relative to fetuses between saline or uninjected controls; and (c) fetuses between two siblings behaviorally activated by the opioid agonist U50,488 also showed less activity and specific behavioral changes compared to controls. Our findings suggest that rat fetuses are directly impacted by sibling motor activity, and thus that a rudimentary form of communication between siblings may influence the development of fetuses in utero.

KEYWORDS
fetal behavior, motor development, *Rattus norvegicus*, sibling effects, somatosensation

1 | INTRODUCTION

Most species of mammals give birth to multiple offspring. Unlike the embryos of birds and nearly all reptiles, which remain encased in separate eggs, the fetuses of polytocous mammals grow together in close proximity, separated only by the thin membranes of the amnion and chorion. The propinquity of siblings during prenatal development introduces possibilities for interaction that are not afforded by the physically isolated chambers of eggs. Placental insufficiency resulting from competing growth in twins (Manning, 1995) and freemartins or more subtle masculinization of females caused by exposure to male siblings' androgens before birth (Forger et al., 1996; Kawata, 2013; Meisel & Ward, 1981; Ryan & Vandenbergh, 2002; Vom Saal & Bronson, 1980) are well known examples of passive interactions between siblings. However, far less is known about direct, active interactions between siblings in utero.

There are many opportunities for interactions between siblings before birth or hatching, and their potential for influencing development are great. Interactions between avian embryos in the same clutch of eggs, or between embryos and incubating parents, often are mediated by vocal communication. Around the time of pipping, an early stage in the process of hatching, embryos of pelicans, grebes, coots, and gulls vary their rate of vocalization within the egg as a function of body temperature, thereby signaling their need for parents to adjust their incubation behavior (Brua, 1996; Bugden & Evans, 1991; Evans, 1990; Evans, Whitaker, & Wiebe, 1994). Among precocial bird species, embryonic vocalizations can stimulate higher metabolic rates, resulting in more rapid growth in eggs laid late in the clutch, and can facilitate the synchronization of hatching (Brua, 2002). Exposure to the vocalizations of siblings in nearby eggs also can foster the development of species-typical auditory recognition in mallard ducks and bobwhite quail (Gottlieb, 1991, 1997; Lickliter & Stoumbos, 1992).

Neither auditory nor visual modalities are likely to contribute to interactions between siblings in mammals, although fetuses are known to hear and respond to vocalizations by the pregnant mother, father, and other individuals outside the womb (DeCasper & Fifer, 1980; Fifer

& Moon, 1995; Kisilevsky, 2016). Chemical signals are a dominant form of mammalian communication after birth, and chemical signals are known to be exchanged between the fetus and mother, as in the coordination of parturition (Nathanielsz, 1994). However, somatic senses are the most likely modality by which fetuses may detect and respond to the behavior of other siblings in utero. Tactile sensitivity is the earliest of the major sensory systems to exhibit function during prenatal development (Gottlieb, 1971). Moreover, fetuses have been shown experimentally to be responsive to cutaneous and proprioceptive cues originating in the environment as well as in their own activity (Brumley & Robinson, 2010; Robinson, 2016; Robinson & Kleven, 2005; Robinson & Smotherman, 1991; Ronca & Alberts, 1994; Smotherman & Robinson, 1988a). It therefore seems plausible, even likely, that they also would be responsive to the movements of contiguous siblings in utero.

During the last few days of a 22-day gestation, the physical conditions surrounding the fetal rat change dramatically. Amniotic fluid becomes sharply reduced in volume and the placenta retracts to the uterine wall, reducing the free space that separates adjacent fetuses (Brumley & Robinson, 2010). The rat fetus also exhibits rapid growth, further reducing the space available for movement. As the environment grows more constrained, fetal motor activity is expressed at high rates, and becomes more organized and coordinated. Given the sensory and motor abilities of the near-term fetal rat, the possibility that motor activity of one fetus may influence the behavior of adjacent siblings in the uterine environment becomes more pronounced.

The hypothetical sensitivity of fetuses to the movements of intrauterine siblings may represent not only a demonstration of prenatal sensory responsiveness, but also a simple and subtle form of communication. Although the concept of communication in animals has often been confounded with notions of intention and purpose (*cf*., Blumberg & Alberts, 1997), communicative behavior actually is ubiquitous among animals. Based on semiotic analysis, Smith (1977) and Hailman (1977) pointed out that communication logically requires only three elements: An individual (the sender) that generates a disturbance in a medium (the signal), which is detected via a sensory modality by a second individual (the receiver), resulting in a change in the Receiver's behavior. For a signal to qualify as communication, the Sender need not intend, nor even be aware, that the signal is sent (the mouse surely does not intend for its not-quite-silent movements to be detected by the cat). Nor is it necessary for the communicative relationship to be adaptive in the evolutionary sense for both sender and receiver (the prey attracted by the flashing light of the angler fish receives no benefit). Communication can be manipulative, exploitative, cooperative, or merely incidental to the ongoing behavior of one or both parties.

In this study, we addressed the question of whether rat fetuses may exhibit a subtle form of communication by responding to the motor activity of other fetuses in the same pregnancy. In the first experiment, we examined the correlation of motor activity among fetuses occupying different positions within the uterus, focusing on subjects lying adjacent to one another in the same uterine horn, non-adjacent in the opposite uterine horn, and in different pregnancies (as a control). In the second experiment, we used curare to eliminate activity in the two fetuses adjacent to a focal subject in utero, and compared the behavior of the subject between curare-injected siblings with other fetal subjects that were flanked by saline-injected or untreated siblings. In the third experiment, we used U50,488, a kappa opioid agonist drug, to stimulate motor activity in adjacent fetuses relative to saline-injected or untreated controls. Together, these three experiments provide evidence that rat fetuses are responsive to movements of adjacent siblings, which may represent a subtle form of intrauterine communication during prenatal development.

2 | GENERAL METHOD

2.1 | Subjects

Subjects were fetuses of pregnant Sprague–Dawley rats (*Rattus norvegicus*; Harlan Laboratories, Indianapolis, IN) that were time-mated. A total of 21 pregnancies provided 63 rat fetuses on gestational day 20 (E20) that were used as "focal" (observed) subjects in the three experiments of this study. From the same pregnancies, 64 non-focal (manipulated) fetuses were injected (with either drugs or isotonic saline) in the second and third experiment. Multiple fetal subjects within each pregnant rat were used in each trial of each experiment.

To produce pregnancies, groups of three females were housed together with one male for 4 days of breeding in 38 × 48 × 20 cm cages. Each day during the breeding period, vaginal smears were collected and examined from females; the day that sperm were detected was designated as E0, the day of conception. All rats were kept in a 12-hr light/12-hr dark environment with controlled temperature (22 °C) and humidity. All fetal subjects and pregnant mothers were maintained and treated in accordance with the guidelines for animal care established by the National Institutes of Health (Institute for Laboratory Animal Resources, 2011), and were approved by the Institutional Animal Care and Use Committee.

Because this study sought evidence for interactions among siblings, the number of siblings available for potential interaction in each uterine horn was a relevant concern. Singleton fetuses within a horn obviously would have no siblings with which to interact. At least three fetuses in a horn were necessary to find one fetus situated between two siblings, and at least six fetuses were required for two non-overlapping groups of three. Table 1 reports the number of fetuses in the left and right uterine horns for each pregnancy and details to which experiment the pregnancy was assigned. Overall, the pregnancies yielded 7.1 ± 0.49 fetuses in the left horn (mean ± SEM; range = 2–11) and 7.1 ± 0.35 fetuses in the right horn (range = 4–10).

TABLE 1 Number of fetuses in each uterine horn in experiments 1–3

Horn	Expt. 1	Expt. 2	Expt. 3
Left	8 7 9 2 11	8 7 7 7 7 6 5 9	3 9 4 8 5 9 8 9
Right	6 6 7 5 4	8 6 8 7 8 9 9 6	10 5 8 7 9 5 7 8

2.2 | Prenatal preparation

Fetuses were observed on E20 of gestation. To permit direct observation and manipulation of fetal subjects, pregnant rats were surgically prepared by a procedure standard in our laboratory for externalization of the uterus (Smotherman & Robinson, 1991). The pregnant rat first was briefly exposed to general inhalant anesthesia until immobile and insensate. While anesthetized, a chemomyelotomy was performed by injecting 100 µl of 100% ethanol into the spinal cord between the first and second lumbar vertebrae (L1–L2). This preparation results in an irreversible spinal blockade at the low thoracic level, thereby eliminating all sensation within the abdomen and hindlimbs of the female.

After spinal blockade was confirmed, the prepared female was secured in a plastic holding apparatus on her back, held at a 45° angle, and immersed to chest depth in a buffered isotonic saline bath (Locke's solution) warmed to 37.5° C. Both horns of the uterus were externalized into the bath through a midline laparotomy. The female then was allowed to recover from general anesthesia (but not spinal blockade) and to acclimate to the bath environment while resting undisturbed in the bath for at least 20 min. This delay was introduced to ensure that the mother and fetuses no longer showed effects of brief general anesthesia, which generally lasted only 2–3 min. These methods for preparing fetal subjects for behavioral observation allowed for direct observation of fetal behavior, experimental manipulation of fetal subjects, and creation of high-quality video recordings of the fetal subjects' movements and behaviors for further analysis. In all three experiments, fetuses were observed through the uterine wall, which is stretched and becomes transparent during the last days of gestation (Smotherman & Robinson, 1991). Both manipulated and focal subjects remained within the uterus, surrounded by intact embryonic membranes, throughout the period of observation. The condition of both pregnant female and constituent fetuses was monitored constantly during the experiment. All fetuses and pregnant females remained in good physical condition with no indication of restlessness, discomfort, or other overt signs of physiological or behavioral stress for the duration of the procedures.

2.3 | Administration of agonist drugs

In experiments 2 and 3, drugs were administered to some of the fetal rats via IP injection to manipulate fetal motor activity. Injections consisted of 0.05 ml of the drug solution or saline vehicle administered with a 30 ga hypodermic needle, which was inserted through the uterine wall, and embryonic membranes (Smotherman & Robinson, 1985). Each day, new drug solutions were prepared in a saline vehicle from frozen aliquots and warmed in the saline bath to body temperature before injection. In experiment 2, two fetuses per pregnancy were injected with 10 mg/kg of *d*-tubocurarine (curare; Sigma-Aldrich, St. Louis, MO) (Moessinger, 1983; Smotherman & Robinson, 1988b); in experiment 3, two fetuses per pregnancy were injected with 1.0 mg/kg U50,488 (U50; Sigma-Aldrich Research Biochemicals, Inc., Natick, MA), a selective kappa opioid receptor agonist (Smotherman, Moody, Spear, & Robinson, 1993). In both experiments, two additional fetuses per pregnancy were injected with the same volume of 0.9% saline. Because individual fetuses could not be weighed before injection, dosages were calculated based on the average weight of an E20 rat fetus (4.4 g). Stock solutions of drugs were prepared in an isotonic saline vehicle, refrigerated until use, and warmed to body temperature (37.5 °C) before administration.

2.4 | Video recording and behavioral coding

Video recordings were collected in experiment 1 to permit repeated playback and observation of simultaneous behavior in different focal subjects. The whole litter was video recorded for 30 min, with all subjects remaining in utero, at 30 fps from a camera providing an overhead view. The two horns of the uterus were positioned within the focal plane of the camera, and external cool lights from fiber optic sources were adjusted to minimize glare and ensure that several fetuses were visible through the uterine wall. Uterine position was actively monitored throughout the session. Fetal movement was coded during video playback using custom event-recording software written by SRR. Fetal movements were coded based on the part of the body engaged in active movement (e.g., head, mouth, body trunk, forelimbs, or hindlimbs). Activity data were summarized across the 30-min observation session for subsequent analysis.

In experiments 2 and 3, fetal movements were coded directly by an observer in real-time. Observation and movement coding of the focal subject began 5 min after drug injection to provide sufficient time for the drugs to exert behavioral effects on the manipulated fetuses. The behavior of focal subjects was observed in utero during a 15-min session in both experiments. Each instance of fetal movement involving head, forelimbs, or hindlimbs was dictated verbally by the observer to a second researcher, who entered the behavioral code into real-time event recording software. Each entry was time-stamped with precision to the nearest 0.1 s.

2.5 | Experimental design & data analysis

In all three experiments of this study, three fetuses were selected from each pregnancy to serve as focal subjects for behavioral observation. To address specific analytic and experimental needs, these subjects were selected based on their uterine position, and relative position to each other. In all cases, two subjects were selected from one uterine horn and the third from the opposite uterine horn. More details about the selection of focal subjects in each experiment is provided in the specific methods below.

Multiple fetal subjects were observed in each pregnancy. To avoid conflation of group effects and litter effects, no more than one subject from each pregnancy was assigned to a particular condition or experimental group (Holson & Pearce, 1992). In experiment 1, groups were defined by spatial proximity, and video playback permitted all groups to be observed, in effect, simultaneously. In experiments 2 and 3, three successive observation sessions were conducted in each pregnancy. Note that the order of testing experimental conditions was

not counterbalanced. Rather, to avoid any possibility of systemic distribution of drugs that might influence fetuses in control groups, the drug-exposure condition was always conducted last of the three 15-min observation sessions. Observation commenced 5-min after injection of saline or drug to provide sufficient time for drugs to take effect (Andersen, Robinson, & Smotherman, 1993; Smotherman et al., 1993).

Frequency counts in different movement categories were used as the primary measures of fetal behavior. Counts also were summed across categories to provide a measure of overall motor activity. In experiment 1, correlations of fetal activity across the 30-min session and probabilities of co-occurrence within 1 s were calculated from pairwise comparisons of fetuses in different uterine configurations. Frequency counts, correlations, and probabilities were analyzed by analysis of variance (ANOVA). Independent variables were the uterine position of subjects (experiment 1) and the drug exposure condition of adjacent fetuses (experiments 2 and 3). Post hoc comparisons of means following overall main effects were performed by Fisher's PLSD. An alpha level of $p < .05$ was used to judge statistical significance.

3 | EXPERIMENT 1

If fetuses are responsive to the movement of their adjacent siblings in utero, then motor activity of adjacent fetuses should be more closely related in time than activity of non-adjacent fetuses. We predicted that movements of one fetus would provoke reactions from adjacent siblings, and evaluated this prediction in three measures; (a) the correlation between rates of motor activity in two siblings; (b) a quantitative profile of the temporal synchrony of movement; and (c) the conditional probability of movement of a particular fetus within a criterion interval—<2.0 s—after movement of a sibling.

3.1 | Method

Five pregnancies each provided three fetuses to serve as subjects for behavioral observation in experiment 1. The three focal subjects comprised two adjacent fetuses in one uterine horn and one non-adjacent fetus in the other horn. Fetuses were selected for observation based on the number and configuration of fetuses in both horns, and on their continuous visibility to the observer. However, fetuses in positions nearest the ovarian end of the uterus were never selected for observation, as this uterine position is more likely to exhibit placental insufficiency and growth retardation (Smotherman & Robinson, 1988b). All three focal subjects were video recorded simultaneously in a 30-min session and behavior was coded during repeated playback, observing just one fetus at a time.

The three focal subjects were selected based on their relative position in the uterus. Two fetuses were directly adjacent to each other in the same uterine horn; the third was in the opposite uterine horn, and thus was spatially separate. One of the two fetuses in adjacent positions was selected randomly, without regard to overall activity, as the reference subject. The fetus in the uterine position adjacent to the reference subject was designated as contiguous; the third observed fetus, in the other horn of the uterus, was designated as opposite. To compare movement synchrony relative to the reference subject, and to provide a control subject that could not be influenced by maternal physiology or any other siblings, the contiguous subject from a different pregnancy was designated as the outgroup control. Each contiguous subject served as the outgroup control for only one other pregnancy.

During video playback, all fetal movements in the five categories of head, mouth, trunk, forelimb (left or right), and hindlimb (left or right) were coded. Fetal activity during observation sessions was summarized with different temporal resolution for different statistical analyses. General fetal activity was summed in each behavioral category over the entire 30-min session. Movements were parsed into successive 15-s bins to compute correlations between time series of different focal subjects in the same pregnancy. Measures of inter-individual synchrony and the probability of co-occurrence of fetal movement involved analysis of inter-event intervals with a precision of 0.1 s.

Frequency counts in each behavioral category and overall activity (summed across all categories) were tallied to provide a general description of rates of fetal activity in each of the different uterine positions. Frequency counts of overall activity also were used to calculate Pearson product-moment correlations across the 120 15-s bins of the time series in three pairwise comparisons within each pregnancy: contiguous versus reference, opposite versus reference, and outgroup versus reference. Frequency counts of fetal movement and correlations of fetal activity were compared across the three positional categories in a series of one-way ANOVAs.

Finally, measures of inter-event synchrony and the conditional probability of movement was used to examine the temporal association of movement of three positional categories (contiguous, opposite, and outgroup) relative to the reference subject in each pregnancy. All intervals between the movement of a focal subject (contiguous, opposite or outgroup) and the closest subsequent movement of the reference subject were computed. The distributions of these intervals, from 0 to 2.0 s, provided profiles of inter-event synchrony between fetuses in different spatial relations. To directly assess the contingency of movement between two subjects, the conditional probability of movement—the likelihood that, given the movement of another focal subject, that the reference subject also move—was calculated for each pairwise comparison in each pregnancy (contiguous, opposite, or outgroup). Further details about the calculation of these probabilities are provided below. Conditional probabilities then were compared by one-way ANOVA. In all statistical analyses, the alpha level was set at $p < 0.05$.

3.2 | Results

A series of two-factor ANOVAs (three uterine Positions × 6 5-min intervals, with the intervals factor treated as a repeated measure) was

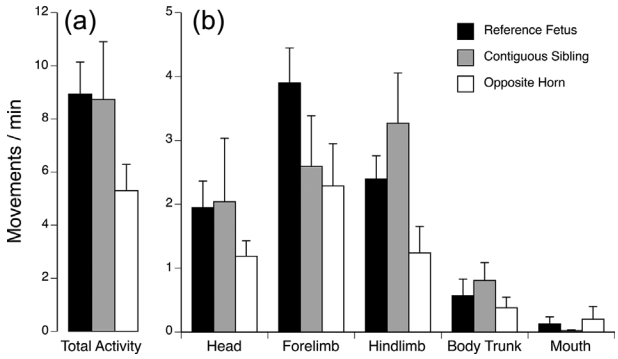

FIGURE 1 Motor activity of rat fetuses in experiment 1. Two fetuses were observed in the same uterine horn (reference and contiguous) and a third fetus in the opposite horn. Bars show mean number of movements overall and in five behavioral categories per min during the 30-min observation session; vertical lines show SEM

conducted to determine if there were any behavioral differences among subjects based on relative position in the uterus. We found no significant main or interaction effects for any behavioral category in rates of movement (all *p*s > 0.05). Mean rates of movement (and SEM) in each behavioral category are depicted in Figure 1.

Although Figure 1 appears to show that absolute rates of movement varied among different uterine positions, variability also was relatively high, with coefficients of variation ranging from 30% to 224%. One apparent pattern was for lower rates of movement in the opposite uterine horn relative to reference and contiguous fetuses. To examine whether uterine horn affected movement rates, additional analyses were conducted with just two uterine positions (reference horn vs opposite horn), with both reference and contiguous subjects contributing data to the reference horn. Despite the doubling of sample size in the reference horn (n = 10), none of these comparisons, with one exception, revealed significant differences in movement rates between the horn containing the reference and contiguous subjects and the Opposite horn. Only the Hindlimb category of movement showed evidence of a significant difference between horns, $F(1,13) = 5.4$, $p < 0.05$.

During our observation of fetal behavior in different subjects within the same litter, it was apparent that two fetuses often moved in close temporal proximity to each other, as though the movement of one fetus stimulated its sibling to respond. As an initial attempt to determine whether activity patterns were related between siblings, we summarized overall motor activity in successive 15-s bins during the 30-min observation session. We then calculated the correlation of activity rates across the 120 time bins in three pairwise comparisons involving the reference subject and its contiguous sibling, the reference subject and its sibling in the opposite horn, and the reference subject with an unrelated fetus in another pregnancy (outgroup). Correlations were calculated separately for each of the five pregnancies that provided subjects in experiment 1. Figure 2A presents a bubble plot depicting the distribution of activity levels, collapsed across all five pairings of reference–contiguous fetuses. The area of each bubble depicts the number of observed intervals; note that most intervals involved low rates of movement in both fetuses.

Three of the five correlations (one per pregnancy) between activity patterns of reference–contiguous fetuses were significant (t values > 1.7, ps < .05; the average correlation (n = 5) was r=.149) (Figure 2B). In contrast, none of the correlations between reference–opposite or reference–outgroup fetuses were significant. A one-way ANOVA that compared correlation coefficients for the three pairings in all five pregnancies indicated that correlations in the reference–contiguous pairing were significantly greater than in

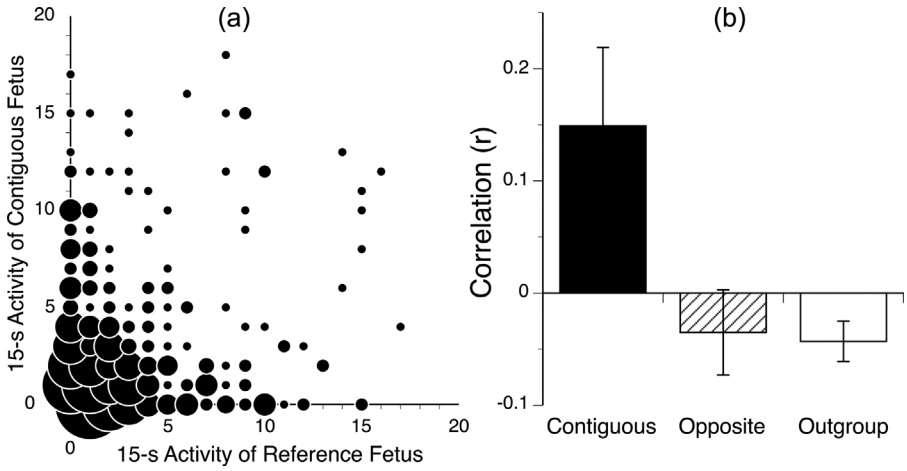

FIGURE 2 Correlated activity of fetuses in different relative positions in the uterus. (a) Bubble plot depicting observed activity of reference and contiguous fetuses in 15-s intervals over the 30-min observation session; the area of each point depicts the relative number of intervals observed. Observations are collapsed across all five pairs in the reference–contiguous condition. (b) Pearson product-moment correlations (r) of activity in 15-s intervals across 30-min time series among fetuses in contiguous, opposite, or outgroup positions. Bars show group means for the five pairs of fetuses in each condition; vertical lines depict SEM

reference–opposite or reference–outgroup pairings, $F(2,12) = 5.6$, $p = 0.019$ (Figure 2B).

The correlational analysis indicated a weak relationship between activity patterns of siblings in adjacent uterine positions, implying that fetuses may influence the behavior of their neighbors in utero. However, fetuses are spontaneously active and many endogenous and exogenous factors can influence rates of movement. To characterize the temporal relationship in activity more precisely, we used a quantitative measure of inter-event synchrony. We have previously reported this method to describe the degree of synchronization of multiple limbs of the same fetal or neonatal subject during spontaneous motor activity (Kleven, Lane, & Robinson, 2004; Robinson, Blumberg, Lane, & Kreber, 2000). In the present study we followed a similar approach to describe the frequency and temporal contiguity of movements of fetuses in different relative positions within the uterus. Each observation period provided a 30-min time series of fetal activity for a focal subject. The timing of occurrence of movements in two different time series was examined in three pairwise comparisons involving the reference subject and its contiguous sibling, the reference subject and its sibling in the opposite horn, and the reference subject with an unrelated fetus in another pregnancy (outgroup). In this analysis, a synchronous movement occurred when movement by one fetus (e.g., reference) was followed within 10 s by movement of the other fetus (e.g., contiguous). All instances of synchronous movement were noted and the frequency of these synchronous movements was summarized by the temporal delay (inter-event interval) between movements.

As shown in Figure 3A, movement synchrony between different fetal subjects was most pronounced with inter-event intervals of less than 2 s, and drops off sharply at longer intervals. A two-factor ANOVA (three positional Pairings × 11 1-s Intervals, with the Intervals factor treated as a repeated measure) was conducted to compare the temporal profiles of movement synchrony in reference–contiguous, reference–opposite, and reference–outgroup pairings. This analysis did not show a main effect of Pairing ($p > 0.20$), but did indicate a significant main effect of Intervals, $F(10,120) = 28.6$, $p < 0.001$, and the interaction of Pairing × Intervals, $F(20,120) = 2.0$, $p = 0.011$. Post hoc comparisons of synchrony, collapsed across Pairings, revealed the main effect of Intervals was entirely confined to the two shortest intervals (0 and 1 s), which differed from one another and from all subsequent intervals (Fisher PLSD, $ps < 0.05$). No other time differences were evident. However, a series of one-way ANOVAs to examine the main effect of Pairing within each time interval found no significant differences in synchrony at any interval. The lower rates of synchrony reflected in the reference–opposite and reference–outgroup pairings logically represent rates of synchronous movement due merely to chance coincidence. At the minimum, the significant interaction provides weak evidence that synchrony at short intervals was more pronounced in the reference–contiguous pairing. This temporal profile indicates that synchronized movements between two fetuses in adjacent uterine positions is confined to inter-event intervals of less than 2 s.

Both the correlational analysis and the profiles of synchronous movement indicated a weak relationship between the temporal patterns of activity in two adjacent fetuses. But both methods are relatively coarse metrics that may have been limited by the small sample size. A more precise metric of the potential interactive effects between siblings is provided by comparing the conditional probability of coincident movement, specifically, the proportion of movements by the reference subject that are immediately preceded by movement of another fetus (in contiguous, opposite, or outgroup positions). The conditional probability $P_{A|B}$, that is the probability that event A will occur given that event B has just occurred, is defined as $P_{A|B} = P_{A\cap B}/P_B$. As a conservative measure based on the synchrony profiles, we

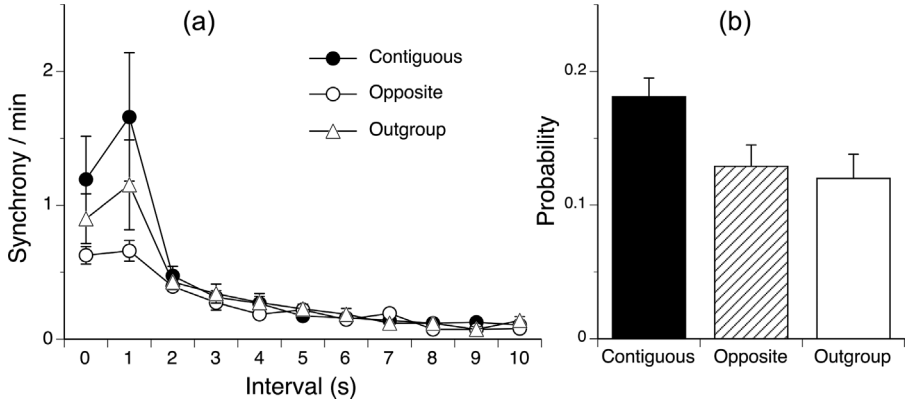

FIGURE 3 Two additional measures of nonrandom association of movement by fetuses in different relative positions in the uterus. (a) Profiles of synchronous movement for fetuses in three positional pairings: adjacent uterine positions (contiguous), different uterine horns in the same pregnancy (opposite), and different pregnancies (outgroup). Points show the frequency of movement events by both fetuses per minute at each of the eleven 1.0-s inter-event intervals; vertical lines show SEM. Note that fetuses in contiguous positions are significantly more likely to move at nearly the same moment (<2 s) than could be accounted for by chance coincidence (outgroup controls). (b) The conditional probability of movement by the reference fetus within <2 s of another fetus in a different uterine position (contiguous, opposite, or outgroup). In both graphs (a) and (b), bars show group means for the five pairs of fetuses in each condition; vertical lines depict SEM

considered a movement by the reference subject to be coincident (A∩B) only if it occurred < 2 s after the movement of another fetus. All movements, regardless of behavioral category, were included in this analysis. All probabilities were calculated per unit time, that is, per second over the course of the 30-min observation session.

The conditional probability of coincident movement, $P_{A|B}$, was calculated as the number of coincident movements of the reference fetus and another subject, $N_{A\cap B}$, divided by the total number of movements by the other subject, N_B. This probability was calculated separately, in each pregnancy, for other fetuses in contiguous, opposite, and outgroup relation to the reference subject.

Average values of $P_{A|B}$ associated with movements by fetuses in contiguous, opposite, or outgroup positions are shown in Figure 3B. Conditional probabilities of coincident movement were analyzed in a one-way ANOVA, which indicated a significant difference among the three relations, $F(2,12) = 4.4$, $p < 0.05$. Post hoc comparisons (PLSD) revealed that $P_{A|B}$ of contiguous fetuses was significantly higher than both other groups. Conditional probabilities did not differ between subjects in opposite uterine horns and outgroup controls. Because there can be no causal connection between fetuses observed at different times in different pregnancies, the reference–outgroup probability represents an estimate of chance coincidence. Overall, reference fetuses were 45% more likely to move immediately after the movement of a contiguous sibling ($P_{A|B} = 0.181$) than after movement of control subjects in the opposite uterine horn or in another pregnancy (outgroup) (average $P_{A|B} = 0.125$).

3.3 | Discussion

The analyses summarized in experiment 1 provide different quantitative metrics of the temporal relationship of motor activity between two adjacent fetuses in utero. Fetal activity is variable, and fetuses in all different uterine positions may show low or high rates of movement. Close examination of specific behavioral categories suggested that hindlimb movements might occur more often in the uterine horn containing the reference and contiguous subjects than in the opposite uterine horn. There would seem to be no a priori reason to expect different activity rates in the two horns. However, the two horns often show a discrepancy in the number of constituent fetuses; we have previously reported that a discrepancy of four or more fetuses is evident in 34% of pregnancies (Smotherman & Robinson, 1988b). By necessity, larger horns are necessary to provide multiple fetal subjects (see Table 1). It is possible, therefore, that the apparent difference in rates of hindlimb movement is related to the number of siblings in the same uterine horn, with more activity related to more siblings in close proximity.

The quantitative analyses (correlation of movement rates, synchrony profiles, and conditional probabilities of movement) are all in agreement that fetuses in adjacent uterine positions are more likely to move in close temporal association than fetuses that are not adjacent. The effect of sibling movement is evidently brief; the strongest evidence from experiment 1 is provided by movements by two fetuses separated by less than 2 s. Fetuses might respond to siblings after longer delays, but given that fetuses move, on average, once every 6 s, a much larger sample would be needed to detect such responses. Our finding that short intervals between movements of different fetuses are much more frequent than long intervals also is entirely consistent with previous reports of temporal patterning of behavioral activity, which often follows an exponential survivor function analogous to radioactive decay (Fagen & Young, 1978; Kleven et al., 2004; Robinson & Smotherman, 1988). Finally, it is important to note in these analyses that absolute rates of synchrony are meaningless unless compared to rates that logically can be attributed solely to chance, such as the synchrony between fetuses in different pregnancies. Because correlations, synchrony, and conditional probabilities of reference and contiguous fetuses were all elevated relative to opposite and outgroup subjects, experiment 1 provides strong empirical evidence that motor activity of fetuses in close uterine proximity is not independent.

A lack of independence in temporal patterns of movement is suggestive, but not conclusive, of direct interactions between adjacent fetal subjects. Fetuses in the same uterine horn share more than spatial proximity. They also share exposure to biochemicals of maternal origin or derived from siblings toward the cervical end of the uterus —"upstream" in terms of the caudal-to-rostral direction of uterine blood flow (Del Campo & Ginther, 1972; Meisel & Ward, 1981). All fetuses in the same pregnancy also are exposed, in principle, to the same stimulus events stemming from maternal behavior or physiology or events in the external environment. Correlation does not prove causation, and it is the causal relationship between movements of adjacent fetuses that we sought to address in experiments 2 and 3.

4 | EXPERIMENT 2

If movements of one fetus increase the likelihood of movement by an adjacent sibling, then changes in the rates of activity of one fetus should be reflected by concomitant changes in the activity of its neighbor. In experiments 2 and 3, we attempted to manipulate the motor activity of fetuses immediately contiguous to a focal subject by direct administration of drugs that either suppress or stimulate motor activity. In experiment 2, we administered curare, a selective drug that blocks transmission at the neuromuscular junction, to completely eliminate movements in the two fetuses adjacent to a focal subject. The rate of activity of the focal subject then was observed and compared to activity of other subjects bounded by siblings that received a control injection of saline or no treatment.

4.1 | Method

Eight pregnant rats provided a total of 72 fetuses on E20 of gestation to serve as observed and manipulated subjects in experiment 2. Only pregnancies that presented at least six fetuses in one uterine horn and at least three fetuses in the opposite horn were used in this experiment. Three experimental conditions were defined based on treatment administered before behavioral observation: SAL, CUR, and

NT. Three fetuses were designated as observed subjects, with the two fetuses immediately contiguous to them (total = 6) designated as manipulated siblings. Treatments were administered only to the manipulated siblings; observed subjects were not directly contacted. Manipulated siblings in the CUR condition were treated with 10 mg/kg of d-tubocurarine, delivered in a 50 µl IP injection. Manipulated fetuses in SAL condition received a 50 µl injection of 0.9% saline. NT fetuses were not treated or otherwise disturbed.

In dozens of prior studies, we have never found effects based on order of testing or position within the uterus, although we have typically counterbalanced these factors in experimental designs. The sole exception has been fetuses in the terminal position closest to the ovaries, which are prone to growth retardation (Smotherman & Robinson, 1988b). In this experiment, observed fetuses were selected from specified positions within the uterus: SAL in the 2nd position within the smaller horn (counting from the ovarian end), CUR in the 5th position within the larger horn, and NT in the 2nd position in the larger horn. Thus, the positions of Manipulated siblings also was determined: SAL (1st and 3rd), CUR (4th and 6th), NT (1st and 3rd). The arrangement of Observed and Manipulated fetuses is illustrated in Figure 4. Moreover, the order of testing was the same in all pregnancies: SAL, CUR, NT. This order was followed to guarantee that one of the control conditions (SAL) could not possibly be influenced by curare entering into general circulation. Because uterine blood flows predominantly caudal to rostral (from the cervical end toward the ovarian), this combination of order and position also maximized the likelihood that the other control condition (NT) would be influenced by any drug that entered general circulation, potentially influencing fetuses other than those that received IP injections. In this design, behavioral differences between SAL and NT Observed subjects would provide evidence of order effects, systemic action of curare, or some combination of these factors.

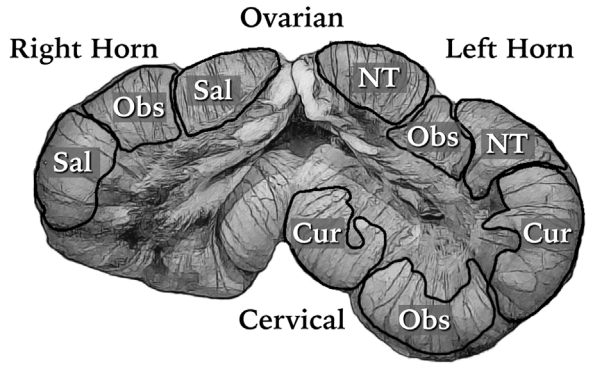

FIGURE 4 Schematic diagram from a photograph of a rat uterus from a ventral perspective depicting relative uterine positions of manipulated and observed fetuses in experiment 2. The positions of six fetuses are outlined. (Differences in apparent size and shape are due to variations in fetal and uterine orientation.) Two manipulated fetuses immediately contiguous to each of three observed subjects were treated by IP injection of saline (Sal) or curare (Cur), or received no treatment (NT), 5 min before each 15-min observation. The same experimental design was used in experiment 3, with curare replaced by the opioid agonist, U50,488 (U50)

Behavioral observation commenced 5 min after treatment of manipulated siblings. All behavior involving gross movement of the head, forelimbs or hindlimbs of observed subjects was coded in real time during a 15-min observation session. Frequency counts in each category were summed over the entire session; Total Activity was calculated as the sum of events across all categories. An additional derived measure, % hindlimb, was calculated by expressing hindlimb movements as a percentage of Total Activity. We have found this derived category to be particularly sensitive to changes in the relative distribution of activity among regions of the body (Robinson & Smotherman, 1992a, 1994). All categories were compared across experimental conditions by one-way ANOVA.

4.2 | Results

Total fetal activity over the 15-min session was analyzed in a one-way ANOVA (three Conditions), which indicated a significant main effect, $F(2,21) = 11.4$, $p < 0.0005$. Post hoc comparisons confirmed that fetal activity was significantly reduced in the CUR group relative to both SAL and NT ($ps < 0.05$). The two control groups did not differ from each other ($p > 0.05$). Fetal activity after SAL, CUR, or NT treatment is illustrated in Figure 5.

Similar ANOVAs examined events in specific behavioral categories. The analyses of head and forelimb movements found no differences among experimental conditions. However, comparison of hindlimb activity revealed the significant effect of sibling treatment, $F(2,21) = 17.0$, $p < .0001$. Hindlimb movements were significantly depressed in CUR subjects bordered by fetuses immobilized with curare relative to fetuses in SAL or NT groups. However, no difference was found in Hindlimb activity between the two control groups ($p > 0.05$). Analysis of % hindlimb similarly showed a significant effect of sibling treatment, $F(2,21) = 3.5$, $p < 0.05$. Post hoc comparison of group means indicated that CUR subjects showed a lower percentage of hindlimb movements than subjects in the NT control condition.

4.3 | Discussion

The rationale for experiment 2 was to suppress motor activity in a pair of fetuses through administration of curare to determine the effect, if any, on the behavior of contiguous, intercalary siblings that received no injection. Our findings reveal that overall motor activity is sharply reduced relative to control subjects bounded by saline-injected or untreated siblings. This effect is driven principally by fewer movements of hindlimbs, which are reduced in CUR subjects 57–62% relative to control fetuses. These behavioral differences are unlikely to stem from direct exposure to the movement-suppressing effects of curare, because NT subjects, located downstream and observed after CUR fetuses, showed undiminished activity relative to SAL controls, which were observed before curare administration. Similarly, behavioral differences were unlikely due to the potential effects of order of testing, given that SAL fetuses were always tested first and NT subjects

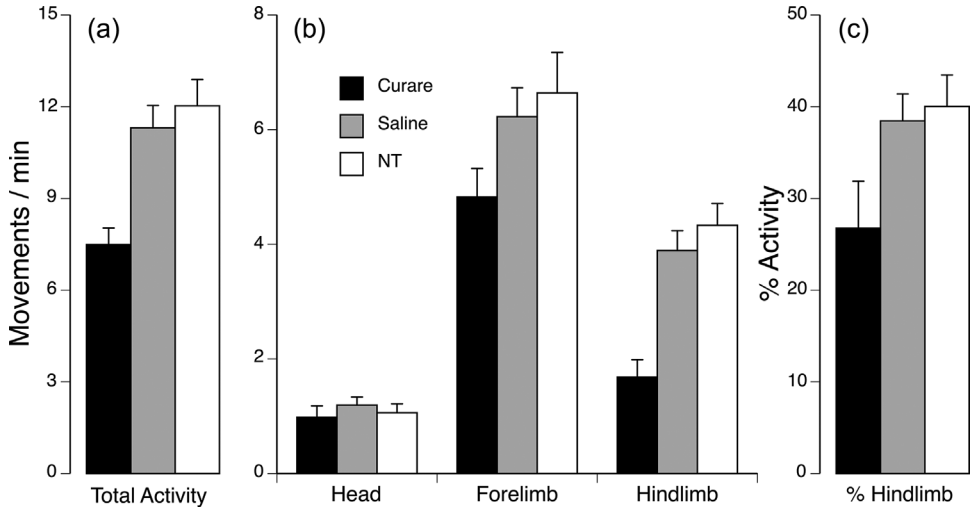

FIGURE 5 Motor activity of observed fetuses in experiment 2. Observed fetuses were not directly treated, but lay between two manipulated fetuses injected with curare or saline, or which received no treatment (NT). Bars show mean number of movements overall (a) and in three behavioral categories (b) per min during the 15-min observation session; vertical lines show SEM. (c) To express changes in the distribution of activity, hindlimb movements also were expressed as a percentage of overall activity

last. It remains possible that some curare diffused across the embryonic membranes to pass from the amniotic compartments of manipulated fetuses to influence the Observed CUR subjects directly. This seems unlikely, however, given the specificity of the behavioral effect: We have no reason to expect that hindlimbs would be selectively responsive to low concentrations of curare exposure. Therefore, we interpret the findings of experiment 2 as consistent with the general hypothesis that changes in the behavior of contiguous siblings can influence the behavior of otherwise unmanipulated fetuses.

5 | EXPERIMENT 3

The results of experiment 2 suggested that reduced activity in contiguous fetuses may result in reduced movement in neighboring siblings. The objective of experiment 3 was to test the opposite effect: Whether increasing motor activity in contiguous siblings might influence the behavior of observed fetuses. We used the drug U50,488 to stimulate motor activity in manipulated siblings. U50,488 is a highly selective agonist of kappa opioid receptors, which we have reported to exert consistent and pronounced effects on fetal behavior. Specifically, administration of U50,488 stimulates a four- to fivefold increase in motor activity, which persists for periods up to 30 min (Andersen et al., 1993; Smotherman et al., 1993). Our prediction was that administration of this activating drug to neighboring fetuses would indirectly stimulate increased motor activity in observed subjects.

5.1 | Method

Experiment 3 followed the same design as experiment 2. Eight pregnancies provided a total of 72 fetuses to serve as observed subjects ($N = 24$) and manipulated siblings ($N = 48$). Fetuses were assigned to three experimental conditions: SAL, U50, and NT. Subjects were tested in the same order, and in the same uterine positions, as described above in experiment 2. Manipulated siblings in the U50 group received 50 µl IP injections of 1.0 mg/kg U50,488, consistent with administration found effective in previous studies of opioid effects on fetal behavior (Andersen et al., 1993; Smotherman et al., 1993). Behavior of observed subjects was coded, quantified and analyzed as in experiment 2.

5.2 | Results

The ANOVA comparing total activity of observed fetuses in the three treatment conditions showed a significant main effect, $F(2,21) = 8.7$, $p = 0.002$. Surprisingly, increased activity of manipulated siblings in the U50 condition resulted in lower levels of activity in observed subjects, compared to both SAL, and NT controls ($ps < 0.05$). The two control groups did not differ (Figure 6).

Head activity also was altered by U50 treatment, $F(2,21) = 5.3$, $p = 0.014$. Post hoc comparisons revealed that head movements were sharply reduced in U50 subjects relative to both SAL, and NT groups ($ps < 0.05$). Forelimb activity also was affected, $F(2,21) = 16.2$, $p < 0.001$, with fewer Forelimb movements expressed in U50 subjects than either of the other groups. Curiously, hindlimb movements were not altered by U50 treatment of adjacent siblings ($p > 0.60$). This suite of effects on different behavioral categories was reflected in a significant effect on hindlimb movements expressed as a percentage of total activity. The analysis of hindlimb % showed a significant effect, $F(2,21) = 9.6$, $p = 0.011$. Post hoc comparisons indicated an increase in % hindlimb relative to both SAL and NT controls ($ps < 0.05$).

5.3 | Discussion

Our expectation in experiment 3 was parallel to that of experiment 2, namely, that increased activity of neighboring fetuses induced by the

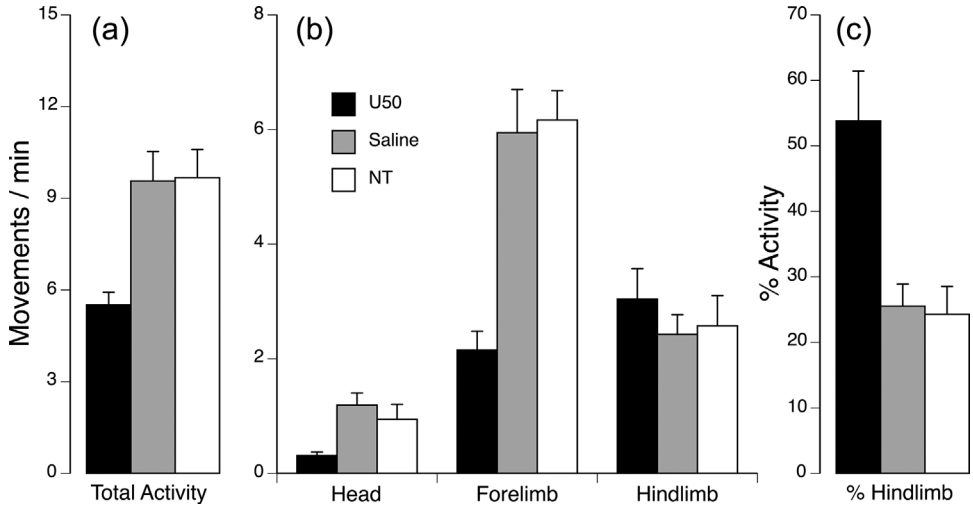

FIGURE 6 Motor activity of observed fetuses in experiment 3. Observed fetuses were not directly treated, but lay between two manipulated fetuses injected with U50,488 or saline, or received no treatment. The three sets of graphs depict overall fetal activity (a), frequency of movement in three behavioral categories (b), and hindlimb movements expressed as a percentage of overall activity (c), as in Figure 5

agonist drug U50,488 would result in increased activity of untreated fetuses between them. The finding that behavior was altered in the U50 group was consistent with an interpretation of sibling interaction, but the reduction (as opposed to increase) in motor activity was counterintuitive. Head and forelimb activity were sharply reduced in the U50 group, while hindlimb movements remained unaffected, resulting in net reduction in total activity and increases in hindlimb %.

As inferred from the results of experiment 2, it is difficult to attribute these behavioral changes to the direct action of U50,488 on observed fetuses. This opioid agonist reliably evokes a sharp increase in hindlimb activity relative to other categories of movement, including head and forelimbs, which is reflected in a dramatic increase in % hindlimb (Andersen et al., 1993; Smotherman et al., 1993). Although % hindlimb also increased in experiment 3, this effect was the result of a decrease in head and forelimb movements, with an overall decrease in motor activity. We have never observed this pattern of effects after direct administration of U50,488, suggesting that the behavioral changes observed in experiment 3 were due to the indirect effects of altered sibling behavior.

6 | GENERAL DISCUSSION

This study reports three experiments, which apply two very different approaches, to answer the question of whether fetuses from the same pregnancy may behaviorally interact with each other. The findings of all three experiments agree that contiguous rat fetuses respond to changes in motor activity in nearby siblings in utero. The first experiment provided three measures of temporally synchronized movement that were significantly elevated among fetuses in contiguous uterine positions relative to noncontiguous siblings or fetuses in other pregnancies. The second and third experiments utilized drugs to suppress or enhance motor activity of siblings on either side of a focal subject. In the second experiment, fetal subjects that lay between two fetuses immobilized with curare showed reduced activity compared to control subjects between saline-injected or untreated siblings. Similarly, the third experiment found reduced overall activity in fetuses between two siblings treated with the opioid agonist U50,488, which produces a marked increase in fetal activity (Andersen et al., 1993; Smotherman et al., 1993). These findings provide strong evidence for direct behavioral interaction between rat fetuses that are developing in the uterine environment together.

Although we believe the evidence supports a conclusion that fetuses are responding to changes in activity of siblings in utero, several possible objections might be raised against that claim. The findings of experiment 1 are correlational in nature, which might suggest that siblings are not responding to each other, but are responding to some unrecognized third variable. It is true that reference and contiguous fetuses share more than adjacent uterine positions; they occupy the same uterine horn and may respond to changes of intrauterine conditions that occur in one horn but not the other. Indeed, we found some indication that total activity, and hindlimb movements in particular, were elevated in reference and contiguous fetuses relative to opposite controls, and higher rates of activity would lead to higher rates of synchronous movements merely by chance association. However, recall that contiguous fetuses also were used as outgroup controls, albeit in different pregnancies; average movement rates of contiguous and outgroup fetuses were identical by definition. Yet rates of coincident movement, as estimated by correlated activity, movement synchrony, or conditional probability, all were elevated in reference–contiguous pairs relative to reference-outgroup pairs.

A second possible objection applies to experiments 2 and 3, where drugs were used to manipulate the motor activity of contiguous

siblings. Drugs administered to manipulated fetuses might have been transported, by diffusion across the membranes of the amniotic sacs or by distribution in maternal circulation, thereby altering the behavior of observed fetuses directly, and not through the intermediary of sibling activity. We believe this possibility is unlikely for three reasons; (1) We have found no evidence in dozens of prior studies that drugs administered directly to a fetus via IP injection affect the behavior of other fetuses in the same pregnancy (Andersen et al., 1993; Petrov, Varlinskaya, Robinson, & Smotherman, 1994, Robinson & Smotherman, 1994; Smotherman et al., 1993); (2) SAL fetuses always were observed before drug administration in the CUR or U50 groups, and NT fetuses were observed last. Moreover, NT fetuses were selected from the same uterine horn as drug-exposed, and closer to the ovarian end of the horn, in the direction of downstream maternal circulation. This experimental design should have maximized the likelihood that NT fetuses were exposed to drugs if the drugs spread beyond the treated fetuses. But the behavior of SAL and NT fetuses did not differ for any variable in either experiment, suggesting that drugs did not escape from the amniotic compartment of treated fetuses in significant concentrations; (3) Curare would be expected to suppress activity if diffusing beyond treated fetuses, and this is consistent with the reduced activity of CUR fetuses in experiment 2, which were surrounded by curare-injected siblings. However, the opioid agonist U50,488 produces a sharp increase in fetal activity, and therefore would be expected to elevate activity if U50 fetuses were directly exposed to the drug. But the overall effect of treating contiguous siblings with U50,488 was to decrease fetal activity in observed fetuses. Only hindlimb movements, expressed as a percentage of overall activity, were elevated. These three points of argument strongly suggest that the behavioral effects observed in experiments 2 and 3 were indeed the consequence of observed fetuses exposed to adjacent siblings with diminished or elevated activity, and not to the direct action of the drugs administered to siblings.

The findings of these three experiments provide strong observational and experimental evidence that siblings developing in the same uterus are responsive to each other's behavior. This responsiveness to sibling behavior is most evident when fetuses reside in contiguous positions within the uterus, suggesting that somatic senses mediate these responses. Indeed, we found no compelling evidence of correlated activity between siblings that were not contiguous. This fact alone argues that fetuses are responding to one another, and not to a common third variable, such as changes in maternal behavior or physiology or events in the external environment.

Why should we find this surprising, or even salient? After all, we have known from the earliest studies of fetal behavior in the early 20th century that fetuses are responsive to tactile stimulation (Angulo y González, 1932; Carmichael, 1934; Narayanan, Fox, & Hamburger, 1971). If a tactile stimulus arises from the movements of a sibling, why should a fetus not respond? It is not the fact of response but the pattern that should capture our attention. Fetuses respond to sibling activity not with a generalized increase in movement, but with specific reactions that are closely timed to the inciting event. Moreover, some patterns of movement are specifically diminished or elevated after changes in sibling activity. Our qualitative observations of unmanipulated fetuses in experiment 1 suggested that fetuses were likely to exhibit hindlimb extensions—kicking—in response to movements by adjacent siblings.

The disproportionate effect of sibling activity on hindlimb behavior was confirmed in both experiments 2 and 3. After adjacent siblings were treated with curare, and therefore were no longer able to move or alter their position in utero, observed fetuses showed a specific reduction in hindlimb activity. After siblings were treated with U50,488, which not only activated siblings but caused them to change their orientation within the uterine horn, hindlimb movements of observed fetuses increased as a proportion of overall activity. Although generalized hindlimb activity is common in fetuses in late gestation, coordinated kicking involving nearly simultaneous extension of both legs is relatively rare (Robinson, 2005). Apart from drug-induced activity or specific behavioral training (e.g., Robinson, 2016), we have observed organized fetal kicking in only one other context: In response to acute compression of the umbilical cord (Robinson & Smotherman, 1992b). As an organized response to hypoxia, such kicking even was effective in occasionally dislodging the vascular clamp that occluded the cord. The efficacy of kicking when faced with umbilical cord compression suggests that vigorous hindlimb extensions exert sufficient force to alter the relative positions of the active fetus and its neighboring sibling. Indeed, recent biomechanical modeling of human fetal movements recorded from cine-magnetic resonance imaging has revealed that fetal kicking generates significant force even at mid-gestation (Verbruggen et al., 2018). Hindlimb extensions therefore may represent a specific behavioral response, and not merely a generalized motor activation, to changes in sibling posture or position.

As mentioned in the introduction, interactions in which a stimulus derived from the behavior of one animal affects the behavior of a second animal can be considered a form of communication. Behavior need not be intentional to serve as a communicative signal (Blumberg & Alberts, 1997). In the present study, movements of fetuses in utero appear to elicit specific behavioral responses from adjacent siblings. This form of interaction is rudimentary, to be sure, but it is no less communicative than the scrambling of infants as different as rabbits and hyenas as they compete for access to a lactating nipple (Bautista et al., 2005; Hofer & East, 2008), or the cycling of rat pups in and out of the core of a huddle within the nest (Alberts, 1978, 2007). Nor should we ignore the possibility that such interactions are adaptive. We have occasionally observed fetuses exchanging intrauterine positions after bouts of elevated activity, and we also have observed incidents where the shift of posture of one fetus partially compresses the umbilical cord of an adjacent sibling. Such interactions must be commonplace in all polytocous mammals, where free space within the uterus is a limited resource (Brumley & Robinson, 2010).

Examples of adaptive fetal behavior have been previously identified, including the stereotypic behavioral response to umbilical cord compression (Robinson & Smotherman, 1992b; Smotherman & Robinson, 1988c), postural adjustments such as human fetuses turning to the vertex position before birth (Suzuki & Yamamuro, 1985; Sival,

Visser, & Prechtl, 1990), adjustment to movement restriction (Robinson, 2005), and self-directed movements such as facial wiping (Robinson & Smotherman, 1991) and self-directed touch (Robinson, Hoagland, Truong, & Mendez-Gallardo, 2016), which may assist in removing obstructions from the nose and mouth. Recognition that some aspects of fetal behavior may be adaptive and not merely the accidental expression of a developing nervous system reinforces an earlier conclusion that behavior is not a trivial aspect of fetal life (Smotherman & Robinson, 1987).

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CONFLICTS OF INTEREST

There is no conflicts of interest to declare.

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