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SYMPOSIUM

Postweaning Isolation Alters the Responses of Auditory Neurons to Serotonergic Modulation

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Synopsis Juvenile social experience, such as social isolation, has profound effects on communicative behavior, including signal production and reception. In the current study, we explored responsiveness to the neuromodulator serotonin as a potential mechanistic link between early life social isolation and auditory processing. The serotonergic system is sensitive to social isolation in many brain regions including the inferior colliculus (IC), an auditory midbrain nucleus. We investigated the effects of social experience on serotonergic responsiveness by measuring cFos, an immediate early gene product, in the IC of female mice. Serotonin was manipulated pharmacologically by administering fenfluramine, pCPA, or saline to mice that had undergone an extreme dichotomy in social experience after weaning: being housed in social groups versus individually. These mice were exposed to a 60-min recording of vocalizations from an opposite-sex interaction and perfused. Using immunohistochemistry, we measured the density of cFos-positive (cFos+) nuclei in the major subdivisions of the IC. Housing condition, drug treatment, and IC subregion all had a significant effect on cFos+ density. The central IC showed the highest density of cFos+ cells and also the most pronounced effects of housing condition and drug treatment. In the central IC, cFos+ density was higher following fenfluramine treatment than saline, and lower following pCPA treatment than fenfluramine. Individually housed mice showed a higher cFos+ density than socially housed mice in both of the pharmacological treatment groups, but not in the saline group. Drug treatment but not housing condition had strong effects on the behaviors of grooming, digging, rearing, and movement. Once the effects of drug condition were controlled, there were no across-individual correlations between cFos+ densities and behaviors. These findings suggest that the responses of auditory neurons to neuromodulation by serotonin are influenced by early life experience.

Introduction

Early life social experience has a profound effect on adult communicative behaviors in a range of animal species (Bottjer and Arnold 1997; Bolhuis and Gahr 2006; George and Cousillas 2013; Prat et al. 2015; Takahashi et al. 2017). A common manipulation of social experience is to house animals individually or socially in early life. Even in species that show little evidence of learned vocalizations, juvenile social isolation can result in changes in the number or duration of calls produced during social interactions (Colonnello et al. 2011; Mahrt et al. 2013; Keesom et al. 2017a; Screven and Dent 2019a). Early life social isolation also influences the perception of species-typical calls. In operant tests of perceptual discrimination, individuals that have been socially isolated in early life may take longer to learn discrimination tasks (mice: Screven and Dent 2019b), or show poorer discriminative abilities (zebra finches: Sturdy et al. 2001; Weisman et al. 2004) than socially reared individuals. Changes in perception as a result of juvenile experience are paralleled by changes in auditory neural responsiveness and selectivity that are biased by the signals encountered in juvenile life (Hernandez and MacDougall-Shackleton 2004; Amin et al. 2013; Hauber et al.

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2013). Altered neural responses can sometimes be explicitly attributed to a difference in the acoustic environment during rearing (Amin et al. 2013), but the quality of the social experience itself can also be influential, as demonstrated when neural changes exist even when acoustic experience is normalized (Cousillas et al. 2006, 2008). Despite the importance of social interaction for perceptual development, how specific neural regulatory systems link social history with sensory processing remains poorly understood.

The serotonergic system is one candidate that could serve as such a link, because it is sensitive to social interaction and can be programmed by the early life environment (Summers 2001; Lapiz et al. 2003; Kiser et al. 2012). Early life social isolation influences the interactions of serotonergic neurons with target neural sites at both presynaptic and postsynaptic levels (Lapiz et al. 2003; Fone and Porkess 2008; Walker et al. 2019). Presynaptically, postweaning isolation often decreases, but sometimes increases, the densities of serotonergic axons (Braun et al. 1999; Whitaker-Azmitia et al. 2000; Lehmann et al. 2003; Kuramochi and Nakamura 2009) or the amount of serotonergic activity during stressful or social contexts (Bickerdike et al. 1993; Fulford and Marsden 1998, 2007; Brenes and Fornaguera 2009; dos Santos et al. 2010; Ago et al. 2013; McNeal et al. 2018). Postsynaptically, isolation can alter the expression of serotonergic heteroreceptors, which are expressed by the non-serotonergic targets of serotonin release (Fone and Porkess 2008). Because the effects of social isolation on responses to vocal signals can be sex-specific, it is also relevant that isolation has sex-specific effects on the serotonergic system (Schiller et al. 2006; Maul et al. 2009; Ross et al. 2019).

In mice, the serotonergic dorsal raphe nucleus (DRN) innervates auditory nuclei via projections from identified subgroups of neurons (Niederkofler et al. 2016; Petersen et al. 2020). One of these auditory regions is a midbrain nucleus, the inferior colliculus (IC), in which both ascending and descending auditory inputs make synapses (Malmierca 2004; Ito and Oliver 2012). DRN-to-IC projections are sensitive to early life social experience. Female but not male mice that are socially housed after weaning have a higher density of serotonergic axons relative to mice that are housed individually (Keesom et al. 2018). Social isolation also influences the dynamics of serotonergic activity. Male mice that have been housed either socially or individually show increases in serotonergic activity during social interaction that are similar in peak level. However, serotonergic increases in individually housed males are more sluggish, and less correlated with social and nonsocial behaviors (Keesom et al. 2017b). Although these findings clearly show that social isolation can alter presynaptic measures of serotonergic function in the IC, whether social isolation also influences the responsiveness of IC neurons to serotonin has not yet been explored.

To assess whether social experience alters the functional effects of serotonin in the IC in female mice, we housed mice individually or socially after weaning. We coupled this with a set of drug treatments in which serotonin was systemically released or depleted, compared with saline. Expression of the immediate early gene product cFos, a marker that is influenced by serotonergic manipulation (Hanson and Hurley 2016), was then measured in the IC following a playback of social vocalizations (Fig. 1). We predicted that individually housed females would show larger effects of serotonergic manipulation than socially housed females. This is because serotonergic receptor expression often shows an inverse relationship to presynaptic serotonergic function, and because our previous work demonstrated that individual housing decreased serotonergic fiber density in the IC of female mice (Keesom et al. 2018).

Materials and methods

Animals

A total of 48 female, non-littermates, CBA/J mice (*Mus musculus*, the Jackson Laboratory, Bar Harbor, ME) arrived post weaning at 21 days of age. Food and water were provided *ad libitum* with a light cycle of 10/14 h day/night. All procedures followed Indiana University's IACUC protocol # 18-025. Female mice were sorted upon arrival into social (i.e., four mice) or nonsocial housing (one mouse) with 24 mice in each social treatment group. Socially housed mice were matched in weight. Animals were in their social treatment groups for 29–30 days before perfusion.

Playback

To provide a salient auditory stimulus, a 60-min playback file was made by repeating a previously recorded 15-min interaction between a male and a female mouse four times (Avisoft-RECORDER USGH software, UltraSoundGate 116H and condenser ultrasound microphone, Glienicke/Nordbahn, Germany). This recording was chosen because it contained many ultrasonic vocalizations (USVs), associated with male courtship of females as well as same-sex interactions (Whitney et al. 1973; Sewell 2009;

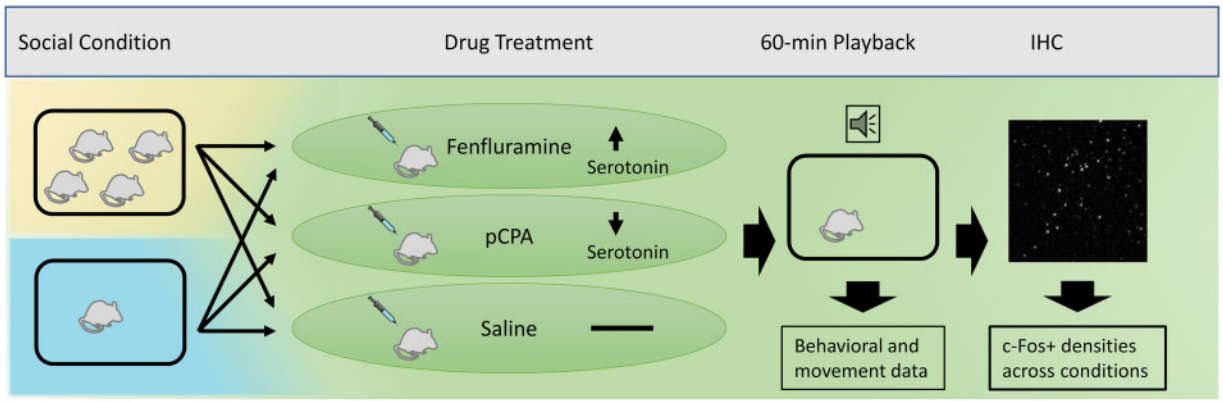

Fig. 1 Conceptual summary of the experimental design. Mice that were exposed to either social or non-social housing were injected with one of three pharmacological treatments, fenfluramine, pCPA, or saline. Mice were then presented with a 60-min auditory playback and perfused. Behavioral and movement data were collected from the playback and cFos+ densities in the IC were measured via fluorescent microscopy.

Hanson and Hurley 2012) that were clearly visible on a spectrogram (Avisoft SAS Lab Pro, Glienicke/Nordbahn, Germany). During opposite-sex interactions, a majority of USVs can be ascribed to males with females producing <20% of the USVs (Neunuebel et al. 2015; Warren et al. 2018). The 15-min segment was high-pass filtered at 30 kHz to minimize low-frequency noise due to movement.

Drug treatment

Socially and individually housed mice were divided into three drug treatment groups. One group received dexfenfluramine HCl (fenfluramine, 10 mg/kg, Sigma Aldrich, St. Louis, MO), an acute serotonin releaser/reuptake inhibitor (Rothman and Baumann 2002). A second group received para-chlorophenylalanine methyl ester (pCPA, 200 mg/kg, Sigma Aldrich), which was administered over the course of 3 days to suppress serotonin synthesis by blocking the rate limiting enzyme tryptophan hydroxylase (Dailly et al. 2006; Ruhé et al. 2007). We have used these doses to elevate and deplete serotonin, respectively, in previous studies (Hanson and Hurley 2016; Petersen et al. 2021). The third group received a control of physiological saline. Each drug treatment group consisted of 16 animals (eight mice from each housing group). Drugs were dissolved in sterile physiological saline and injected intraperitoneally to briefly anesthetized mice (4% isoflurane, SomnoSuite, Kent Scientific, Torrington, CT) at a volume of 0.005 mL/g.

Experiments were run over the course of 4 days. To match the injection schedule among groups, the FEN and saline groups also received saline injections during the first 3 days of pCPA treatment, while the Day 4 injection for the pCPA group was saline. For all groups, the final injection occurred 90 min prior to playback on the fourth day. On Days 1–3, drug injections were performed between 9:00 A.M. and 1:30 P.M. Animals were subsequently allowed to recover in their home cages before being placed in a sound-attenuated chamber for a 45-min acclimation period. Day 4 injections and experimental tasks were performed between 10 A.M. and 5 P.M.

On Day 4, all animals were held in a sound-attenuated chamber for at least 40 min prior to the start of injections to reduce immunohistochemical artifacts from ambient noise in the room. Mice were administered injections individually, placed into a fresh cage, and returned to the holding chamber for 45 min. As reported in Petersen et al. (2021), mice were presented with a 60-min playback and returned to the holding chamber for 30 min prior to perfusion to allow for changes in cFos expression to accumulate in response to neuronal activation. This timeline was chosen to maximize the expression of cFos, which is detectable about 30 min after the onset of a stimulus and peaks after about 90–120 min (Kovács 2008). We have not previously tested a shorter stimulus playback, but playback-induced changes in immediate early gene expression have also been observed in the auditory midbrain of non-mammalian vertebrates after shorter playback exposure (Hoke et al. 2010; Mohr et al. 2018), and in the mouse IC following shorter exposure to "siren-like" sound (Nguyen et al. 2020) as well as in response to longer stimulus presentation regimes (D'Alessandro and Harrison 2014; Ouda et al. 2016).

Estrous phase measurement

Estrous phase was assessed over a 4-day period including the day of perfusion, with the exception of

four mice only assessed on Days 2–4. Estrous phase was determined by the percentages of leukocytes, nucleated epithelial cells, and cornified epithelial cells found in vaginal lavages, as described previously (Goldman et al. 2007; Byers et al. 2012; Keesom et al. 2018). For the purposes of analysis, females were grouped into those in proestrus or estrus versus those in metestrus or diestrus (Hanson and Hurley 2016). Because estrous phases were observed rather than manipulated, they were not perfectly balanced across drug treatments. There were nine pro/estrous females and six met/diestrous females in the saline treatment, eight pro/estrous females and eight met/diestrous females in the fenfluramine treatment, and six pro/estrous females and ten met/diestrous females in the pCPA treatment.

Immunohistochemistry

Mice were euthanized using isoflurane, transcardially perfused with Krebs–Henseleit buffer (pH 7.2) until the runoff was clear (50–100 mL) and then perfused with 4% paraformaldehyde (50–100 mL) using the MasterFlex L/S pump. The brain was removed, placed in 4% paraformaldehyde overnight, and then moved to 30% sucrose until the brain sank (up to 72 h). The brains were sectioned at 50 μm on a freezing microtome into three series and stored in cryoprotectant solution (30% sucrose, 30% ethylene glycol, and 1% polyvinylpyrrolidone in phosphate-buffered saline [PBS]) at −80°C until they were used for immunohistochemistry (IHC). IHC was performed in three groups of 16 animals. Each group contained an equal number of individuals from social and individual housing, and individuals from two different pharmacological treatments. Additionally, brains from each social–pharmacological treatment (e.g., mice housed in groups and administered saline) were split and processed across IHC groups.

IHC was performed over 3 days. Two primary antibodies were run sequentially on the first and second days and secondary antibodies were run on day 3. On Day 1, tissue was rinsed in PBS, blocked (10% Normal Donkey Serum [NDS] and 3% Triton X-100 in PBS) for 1 h, and then incubated overnight at room temperature in rabbit anti-cFos polyclonal antibody in primary antibody (Sigma Aldrich) at 1:2000 dilution (5% NS and 3% Triton X-100 in PBS). On Day 2, tissue was rinsed in PBS with 0.5% Tween 20, blocked for 30 min (50 mM glycine, 0.05% Tween 20, 0.1% Triton X-100, 0.1% BSA, and 2% NDS in PBS), and incubated overnight in GAD 67 antibody (Sigma Aldrich) diluted 1:2000 in Antibody Signal Enhancer Solution (10 mM glycine, 0.5% Tween 20, and 0.1% Triton X-100 in PBS; Rosas-Arellano et al. 2016). On Day 3, the tissue was rinsed with 0.5% NDS in PBS and incubated in the dark for 2 h in Alexaflour 488 Donkey anti-rabbit (3 µL/mL) and Alexaflour 594 Donkey anti-mouse (5 µL/mL) (Life Technologies, Eugene, OR) in diluent (10% NS and 3% triton-x100 in PBS). Samples were then rinsed and mounted using ProLong Gold Antifade Mountant with 4′,6-diamidino-2-phenylindole (DAPI; Life Technologies).

To ensure specific labeling of cFos-positive (cFos+) cells in tissue, a cFos control peptide was used for adsorption of the cFos antibody (Immunostar, Hudson, WI) prior to staining. We compared three conditions: (1) the cFos antibody in diluent (Fig. 2A), (2) the cFos control peptide (5 µg/mL) was pre-adsorbed with the cFos antibody (Fig. 2B), and (3) only diluent (Fig. 2C). All steps for all three groups were run in parallel and incubated overnight at 4°C prior to immunohistochemical staining. Tissue was rinsed and incubated in primary antibodies according to IHC Day 1 above. On the second day, samples were rinsed with 0.5% donkey serum in PBS and incubated in the dark in Alexaflour 488 Donkey anti-rabbit (3 µL/mL) in diluent for 2 h. Samples were then rinsed and mounted.

Image acquisition and processing

Tissue was imaged using an Applied Precision DeltaVision personalDV microscope (GE Healthcare Life Sciences, Marlborough, MA) at the IU Light Microscopy Imaging Center (Bloomington, IN). Montage images of the entire IC (left and right) were captured using an Olympus 10×/0.40 objective lens. Light and camera settings were the same across all images (FITC filter: 50% transmission: 0.4 s exposure/A594 filter: 32% transmission: 0.40 s exposure). Images were taken in a *z* stack, deconvolved, and stitched using Resolve3D softWorX 7.0 program Release RC6 (GE Healthcare Life Sciences). Images were processed in FIJI (Schindelin et al. 2012, 2015; Schneider et al. 2012), and the brightness and contrast were adjusted using identical settings across all images in Photoshop to increase intensity of signal (Adobe Photoshop CC 2019, Version 20.0.0, San Jose, CA).

IC regions (central nucleus, dorsal cortex, and lateral cortex) were identified using The Mouse Brain in Stereotaxic Coordinates (Franklin and Paxinos 2008) as a guide. Regions of interest were created in FIJI by comparing outlines from the guide with GABAergic puncta stained with GAD 67 in Adobe

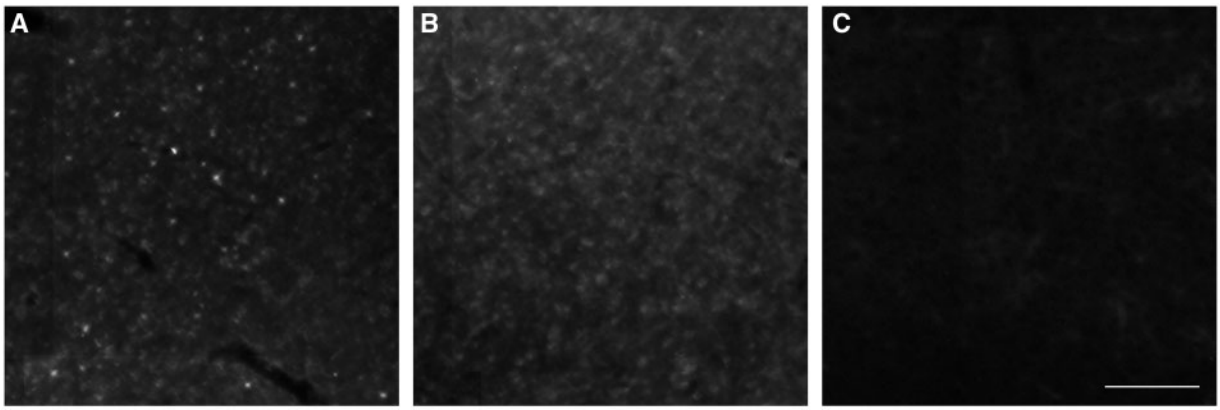

Fig. 2 cFos label in the IC. (**A**) Section showing positive label of cFos+ nuclei in the IC. (**B**) Section incubated with cFos primary preadsorbed with a control peptide. (**C**) Section processed without primary antibody. Scale bar is 150 μm.

Photoshop (CC 2019 Version 20.0.0). cFos+ nuclei were counted by region using the Custom Lab Image Analysis/Processing Macros ImageJ plugin and protocol in ImageJ (Timothy and Forlano 2019). One animal was excluded from image analysis because of poor staining.

Behavioral analysis

Animals were recorded for behavioral analysis during the 60-min playback on Day 4. Behavioral analysis was performed blind to treatment group. Movement tracking was completed with ImageJ manual movement tracker (Bolte and Cordelières 2006; https://imagej.nih.gov/ij/plugins/track/jacop.html). Movement was recorded by selecting the position of the mouse at its tail base (where the tail connects with the body) every 100th frame (every 3.34 s) for the entirety of the 60-min video. The measurement for each drug treatment group was calculated from an average of the ratio distance traveled over time (cm/s). Behavior tracking was completed with OD Log video coder (Macropod Software). Measured behaviors were digging, grooming, rearing, and jumping. Digging was defined as the shuffling of the bedding with at least two limbs. Grooming was defined as brushing the fur with any of the feet. Rearing was defined as vertical movement with both front feet on the walls of the cage. Jumping was counted when the mouse left the floor of the cage with all four legs.

Behaviors were measured by researcher J.M.S. and began when similarity for multiple measurements of the same video were 95%. Behaviors were quantified as the time spent performing behaviors and the number of times the behavior was performed. Because there was a significant difference in total time spent moving between drug treatment groups, behavior times were normalized as a ratio of the time spent performing the behavior against the total time the animal was visible. Any time the mouse was obscured from view and performing any movement with at least its head underneath the bedding was subtracted from the total behavior time.

Statistical analysis

Statistical analyses were performed using Statistica software (Tibco, Palo Alto, CA). Repeated measures ANOVAs were used to assess effects of social housing condition and drug treatment on cFos+ densities in the central, lateral, and dorsal subdivisions of the IC. Factorial ANOVAs were used to assess the influence of social housing, drug treatment, and estrous phase, and the interaction of these factors on behaviors. Separate ANOVAs were performed for each behavior to allow detection of the influence of potentially different categorical factors on different behaviors. Across-individual correlations between cFos+ densities and behaviors were assessed with multiple regressions, with behaviors as dependent variables and cFos+ densities as regressors. General linear models were used to assess the influence of categorical factors and the continuous factor of cFos+ density on behavior, with a parallel model for each behavior. Categorical factors were the social treatment, drug treatment, and estrous phase, and the continuous variable was cFos density in the central subdivision of the IC, the subdivision with the highest overall cFos density. Benjamini–Hochberg corrections for false discovery rate, with a conservative Q value of 0.05, were used to correct for multiple comparisons (Benjamini and Hochberg 1995).

Results

Social isolation influences the effect of manipulation of serotonin on the density of c-Fos+ neurons in the IC

Effects of housing condition and pharmacological treatment on the density of cFos+ neurons were

Table 1 Influence of social condition and drug treatment on cFos+ densities over three IC subregions

Effect	Df	*F*	*P*-value
Soc	1	5.5	0.024*
Drug	2	4.8	0.014*
Socxdrug	2	0.9	0.419
Region	2	236.8	0.000*
Regionxsoc	2	9.8	0.000*
Regionxdrug	4	5.8	0.000*
Regionxsocxdrug	4	0.7	0.620

assessed across three subregions of the IC: the central, lateral, and dorsal subdivisions. This was accomplished with a repeated measured ANOVA with subregion as a within-subjects factor and social and pharmacological treatments as categorical variables (Table 1). Both social condition ($F_{1,41} = 5.5$, $P = 0.024$) and drug treatment ($F_{2,41} = 4.8$, $P = 0.014$) influenced cFos+ density, as did the IC subregion ($F_{2,41} = 236.8$, $P < 0.001$). There were also significant interactions between the IC subregion and both social and drug treatments (Table 1). Contrary to a previous finding (Hanson and Hurley 2016), estrous state did not have a main or interactive effect ($F_{1,35} = 0.56$, $P = 0.46$), nor did it alter whether other conditions were significant (region: $F_{2,70} = 199.44$, $P = 0.00$; housing $F_{1,35} = 4.58$, $P = 0.039$; drug $F_{1,35} = 4.075$, $P = 0.026$), so it was left out of the final model.

Figure 3 illustrates these differences for the three main subdivisions of the IC (panels A–C). In general, densities of cFos+ nuclei were highest within the central subdivision of the IC (Fig. 3A) and lowest within the lateral subdivision (Fig. 3C). Within the central subdivision (Fig. 3A), *post hoc* tests (Fisher's least significant difference) reveal that significant differences ($P < 0.05$) between the socially and individually housed conditions did not occur for saline-treated animals, but were present for both the fenfluramine and pCPA-treated groups (asterisks). For both the fenfluramine- and pCPA-treated groups, females housed individually had higher cFos+ densities. However, cFos+ densities were not identical in these groups; instead, pCPA-treated females showed significantly lower cFos+ densities than fenfluramine-treated females for both the social and individual housing groups. In the dorsal subdivision of the IC (Fig. 3B), the only drug treatment that showed a significant difference between socially and individually housed females was the fenfluramine group. Similar to the central IC, the pCPA-treated

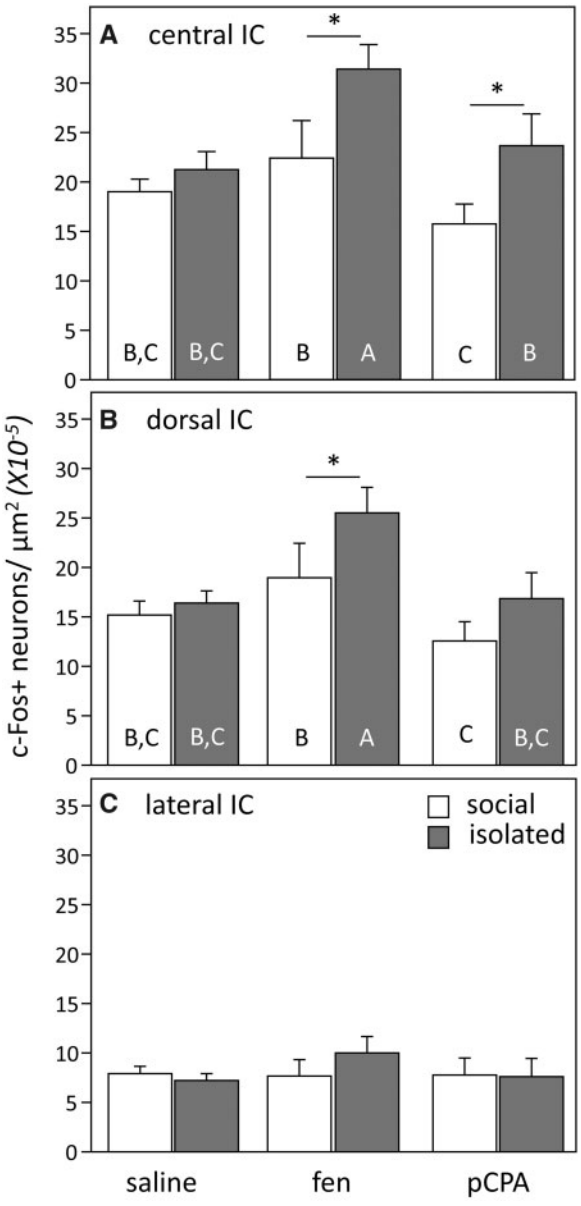

Fig. 3 Densities of cFos+ cells in (**A**) the central subdivision, (**B**) the dorsal subdivision, and (**C**) the lateral subdivision of the IC. Densities of c-FOS+ neurons are shown following treatment with saline, fenfluramine, and pCPA. Open bars indicate social postweaning housing and filled bars indicate individual housing. Letters denote the results of *post hoc* tests; letters only refer to results within a single subdivision, not across subdivisions.

females had significantly lower cFos+ densities than fenfluramine-treated females for both the social and individual housing groups. In the lateral subdivision of the IC (Fig. 3C), there were no significant differences among any of the treatment groups.

Behavior of female mice in response to manipulation of serotonin

The effects of housing condition, pharmacological treatment, and estrous phase on the behaviors of

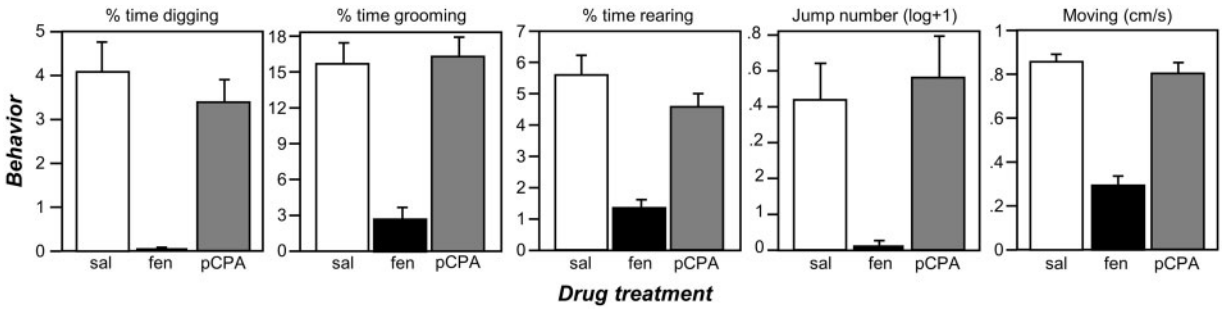

Fig. 4 Effects of saline (sal), fenfluramine (fen), and pCPA on digging, grooming, rearing, jumping, and movement. Fenfluramine decreased all behaviors, but pCPA treatments did not differ from saline.

female mice were also assessed. The measured behaviors were digging, grooming, rearing, jumping, and moving. Because total trial times varied slightly, digging, grooming, and rearing behaviors were measured as a percentage of the total time in the trial. Jumping was measured by number of events, and movement was measured as the average cm/s traveled. The effects of housing condition, pharmacological treatment, and estrous phase were assessed using factorial ANOVAs for each behavior separately, to maximize the ability to detect different relationships to these variables for different behaviors. For every behavior, the pharmacological treatment had a strong effect ($F_{2,35} = 33.35$ for digging, 26.63 for grooming, 25.73 for rearing, 7.39 for jumping, 67.12 for moving, $P < 0.002$ for all behaviors).

Fenfluramine drove the significant effects of drug treatment by drastically decreasing all behaviors relative to the saline group (Fig. 4: decrease by 99.4% for digging, 82.1% for grooming, 75.3% for rearing, 96.9% for log jumping, and 65.1% for movement rate). In contrast, pCPA had less effect on behaviors, decreasing digging by 16.9%, rearing by 17.4%, and moving by 6.2%, and increasing grooming by 3.6% and log jumping by 14.9%. For each behavior, the fenfluramine group strongly decreased behavioral performance relative to the two other pharmacological conditions (one-way ANOVAs, $P < 0.005$ for each behavior), but the saline and pCPA groups were not different ($P > 0.05$ for each behavior).

The only behavior that showed a significant effect of factors other than pharmacological treatment was digging (Table 2). Digging was influenced by the three-way interaction among social condition, drug treatment, and estrous phase. Although the *P*-value for the social treatment was less than 0.05, this was not significant following correction for multiple comparisons. This result suggests multiple state-dependent conditions might combine to determine the influence of serotonergic manipulation on digging behavior.

Table 2 Influence of social housing condition, drug treatment, and estrous phase on digging behavior

Effect	df	*F*	*P*-value
Soc	1	7.26	0.011
Drug	2	33.35	0.000*
Est	1	0.03	0.869
Socxdrug	2	2.59	0.089
Socxest	1	1.72	0.199
Drugxest	2	2.38	0.107
Socxdrugxest	2	7.77	0.002*

Across-individual correlation between cFos expression and behavior

We further assessed whether cFos activity in the IC correlated with behaviors across individuals, because such correlations have been previously observed (Hanson and Hurley 2016). Digging, grooming, and moving were significantly predicted by cFos+ densities in the central, lateral, and dorsal IC subdivisions (multiple regression, digging $F_{3,43} = 5.93$, $P = 0.002$; grooming $F_{3,43} = 3.36$, $P = 0.027$; moving $F_{3,43} = 3.78$, $P = 0.017$). Figure 5 illustrates the relationship between cFos in the central IC versus digging (Fig. 5A), grooming (Fig. 5B), and moving (Fig. 5C). However, these correlations could be driven by the effects of fenfluramine, because fenfluramine both increased cFos+ densities and decreased measures of behavior. We further tested this hypothesis by performing general linear models for each behavior incorporating the categorical factors of drug treatment, social treatment, and estrous phase, and the continuous variable of cFos+ density in the central IC. Although the effect of drug treatment for all behaviors was highly significant, no behaviors were significantly predicted by cFos+ densities (Table 3).

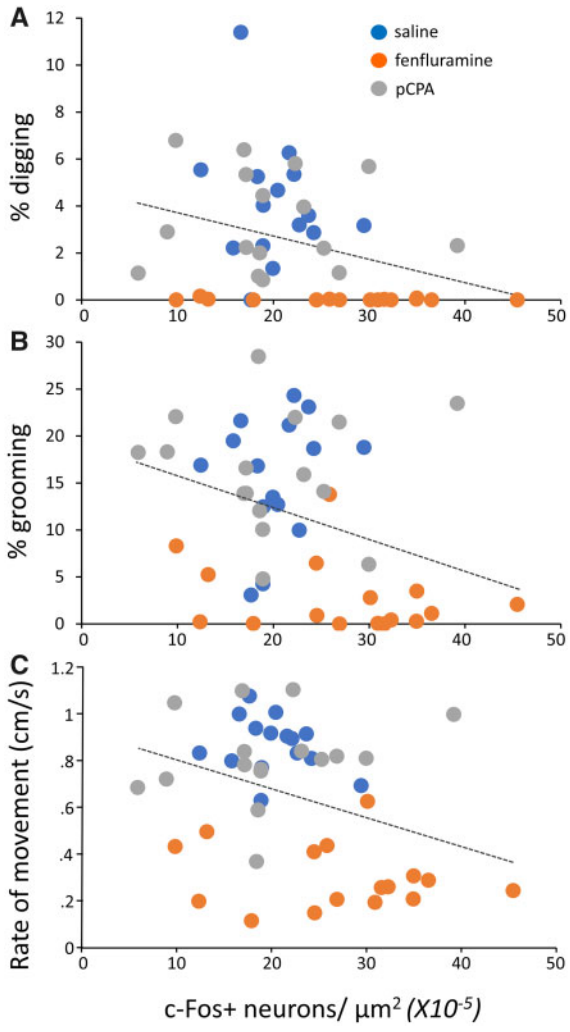

Fig. 5 Across-individual correlations between the densities of cFos+ cells and (**A**) percent time spent digging, (**B**) percent time spent grooming, and (**C**) rate of movement (cm/s).

Discussion

Juvenile social experience has profound effects on communicative behavior, including signal production and reception (Bottjer and Arnold 1997; Bolhuis et al. 2000; Hernandez and MacDougall-Shackleton 2004; Woolley 2012; George and Cousillas 2013; Prat et al. 2015; Takahashi et al. 2017). In the current study, we explored a mechanistic link between social experience and cFos activation in an auditory midbrain nucleus, the IC. This was accomplished by pharmacologically manipulating serotonin in female mice that had undergone an extreme dichotomy in social experience after weaning: being housed in social groups versus individually. We found that both social treatment and serotonergic manipulation influenced the densities of nuclei expressing cFos. These findings suggest that the serotonergic system plays a role in mediating the effects of social experience in a central auditory region.

Social isolation alters the effects of serotonergic manipulation

Social treatments (individual versus social housing) and pharmacological treatments (saline, fenfluramine, or pCPA) both had effects on the densities of cFos+ profiles in the IC. The subregion of the IC also had a strong effect, so that not only were overall cFos+ densities higher in the central subdivision of the IC (Fig. 2), but the effects of social and drug treatments were also more pronounced in this region (Fig. 2A). Within the central IC, there were interesting patterns among and across treatment groups. Among drug treatments, fenfluramine increased cFos+ densities relative to saline, and pCPA-treated mice had significantly lower cFos+ densities than fenfluramine-treated mice. In a previous study, similar effects of fenfluramine and pCPA further depended on the estrous phases of females, with fenfluramine increasing cFos+ densities more, and pCPA decreasing cFos+ densities more, during estrus and proestrus (Hanson and Hurley 2016). In the current study, estrous phase did not have an effect on cFos+ densities.

One potential reason for the difference between the former and current studies is that the statistical and experimental contexts were different, since the current study involved the further dimension of social experience. In addition to the experimental context, our observational approach to estrous phase may also have contributed to the difference between

Table 3 Output of GLMs assessing the influence of categorical (soc, drug, and est) and continuous (centIC) variables on behavior

		Dig		Groom		Rear		Jump		Move	
Effect	**df**	***F***	***P*-value**	***F***	***P*-value**	***F***	***P*-value**	***F***	***P*-value**	***F***	***P*-value**
Centic	1	0.00	0.949	0.003	0.955	0.227	0.636	0.361	0.551	0.318	0.576
Soc	1	1.47	0.232	0.022	0.882	0.415	0.523	0.006	0.940	1.178	0.284
Drug	2	15.94	0.000	22.829	0.000	24.027	0.000	8.471	0.001	49.279	0.000
Est	1	0.17	0.682	2.209	0.145	0.341	0.562	1.062	0.309	0.351	0.557

these two studies. Since we only observed naturally occurring estrous phase, estrous phases were not totally balanced among experimental groups, although the skew was not extreme. However, these factors together may have reduced our ability to replicate a relatively weak and interactive effect of natural estrous phase. A more robust way to assess the effects of estrous phase could be to manipulate ovarian hormones as an experimental variable. For example, exogenous estradiol increases egr-1 expression in the auditory midbrain of female túngara frogs in response to conspecific calls (Chakraborty and Burmeister 2015). In female white-throated sparrows, selectivity of the immediate early gene response for conspecific song, as well as sound-induced serotonin and dopamine turnover, are dependent on the administration of estradiol to induce a breeding-like state (Maney et al. 2006; Rodríguez-Saltos et al. 2018).

Postweaning social isolation influenced the effects of drug treatments in the central IC. In the saline group, there was no difference between the socially and individually housed mice. In both the fenfluramine and pCPA groups, however, mice housed individually had higher cFos+ densities than mice housed socially. The parallel influence of housing condition in these two drug treatments but not in saline is puzzling, given that fenfluramine and pCPA have divergent effects on the serotonergic system, with fenfluramine releasing serotonergic stores and pCPA blocking serotonin synthesis. However, pCPA is most effective when administered over multiple days. This longer-term application could lead to adaptation of postsynaptic elements of serotonergic signaling like receptors (Airhart et al. 2012), which could increase the responsiveness of IC neurons to a reduced level of serotonin release. In addition, all pharmacological treatments were administered systemically. Differential effects of fenfluramine and pCPA at upstream targets could therefore also contribute to the effects of these two drug treatments.

Postweaning social isolation broadly affects interactions between serotonergic axons and targeted brain regions at both presynaptic and postsynaptic levels (Fone and Porkess 2008; Walker et al. 2019; Keesom and Hurley 2020). This range of effects is reflected in serotonin–auditory interactions. Presynaptically, female mice housed socially have a higher density of serotonergic fibers than females housed individually (Keesom et al. 2018). Males from different housing conditions show no difference in fiber density, but do show differences in the dynamics of serotonergic availability measured with carbon fiber voltammetry. Individually housed males show increases in serotonin during a social interaction that take longer to reach their peak and have a lower correlation to social and nonsocial behavior than socially housed males (Keesom et al. 2017b). Our findings therefore parallel a larger body of work on the effects of postweaning social housing on serotonergic modulation.

Little evidence that cFos corresponds to behavior

We found little evidence that the densities of cFos+ nuclei corresponded to behavior. With the exception of digging behavior, the only experimental variable that influenced the behaviors we measured was fenfluramine treatment, which significantly decreased the performance of all behaviors. Although an interaction of social treatment, drug treatment, and estrous phase additionally influenced digging, we have previously observed an effect of call playback on digging behavior (Hanson and Hurley 2016). These findings across studies suggest that digging behavior may be sensitive to some aspects of the social environment. Functional views of digging behavior vary among authors; digging may be interpreted as an indication of an obsessive-like state, or as a normal searching/foraging behavior (Masuda et al. 2000; Odland et al. 2021). Given the association between digging and sniffing behavior in an olfactory discrimination task (Wesson et al. 2008), it is possible that digging is associated with olfactory investigation in some contexts.

Although there were significant across-individual correlations between cFos+ densities and multiple behaviors, these were driven by the effects of fenfluramine in simultaneously decreasing behaviors and increasing cFos+ densities (Fig. 5). The lack of correspondence between cFos+ densities and behavior may be due in part to the profile of behaviors we assessed. These behaviors did not involve explicit responsiveness to the playback, although we have previously reported an effect of playback of vocalizations on digging behavior (Hanson and Hurley 2016). Isolation additionally influences social or anxiety-like behaviors (dos Santos et al. 2010; Walker et al. 2019), which the mice in the current study did not have the opportunity to display.

In contrast, multiple authors have reported a connection between midbrain immediate early gene expression and vocal responsiveness or vocal production in females (Hoke et al. 2008, 2010; Hanson and Hurley 2016). In female túngara frogs, midbrain immediate early gene activation matches preference for male calls, in that gene activation is selective for conspecific over heterospecific calls, a

difference that does not appear in the midbrain of males or at auditory processing levels below the midbrain (Hoke et al. 2008). Moreover, female midbrain activity corresponds to the activation of forebrain networks as well as behavior (Hoke et al. 2010). These findings support a model of the auditory midbrain as a key site for sensorimotor transformation (Hoke and Pitts 2012). Immediate early gene activation in some species also corresponds to self-produced vocal signals. In female white-crowned sparrows, immediate early gene expression in an auditory area correlates positively with calls produced by the females in response to the playback of male songs (Maney et al. 2003). In female mice, the densities of cFos+ neurons in the IC are negatively related to the numbers of broadband vocalizations (squeaks) produced by females during opposite-sex interaction (Hanson and Hurley 2016). In contrast, cFos+ densities have no relationship with the numbers of BBVs that are contained in a playback. Immediate early gene activation in auditory regions may therefore be related to vocal output in addition to received auditory signals.

A correlation between cFos in the IC and behavior could arise from a relatively direct IC-motor system connectivity. It is true that the IC connects relatively directly to some sensorimotor pathways. For example, projections from the IC through the superior colliculus, to cholinergic modulatory regions, are involved in the crucial role of the IC in the prepulse inhibition of the acoustic startle response (Fendt et al. 2001; Li and Yue 2002). The IC also projects to the nearby periaqueductal gray (Goyer et al. 2019), and electrical and chemical stimulation of the IC also triggers defensive behaviors (Brandão et al. 2005). There is therefore ample evidence that some outputs of the IC can trigger or modify specific classes of behavior. However, a correlation between cFos in the IC and behavior does not necessarily indicate a close network connectivity to motor regions. For example, if the information on acoustic signals represented by cFos expression in the IC were particularly salient for a specific behavior, this functional relationship could also underlie a correlation. Both of these possibilities could be explored further in mice by assessing whether the suppression of male USVs by the playback of female squeaks is reflected by cFos expression in the IC.

Modulation of sensory processing

An important role of neuromodulatory systems across vertebrate taxa is the regulation of sensory processing in accordance with multiple aspects of behavioral context and internal state. These are sometimes related to long-term changes in reproductive state. For example, catecholaminergic projections to central and peripheral components of the auditory system in female midshipman fish are seasonally plastic and in the saccule may regulate seasonal auditory sensitivity (Forlano et al. 2015; Perelmuter et al. 2019). In the auditory system of white-throated sparrows, seasonally varying gonadal hormones cause changes in catecholaminergic and serotonergic innervation and turnover in both males and females (Matragrano et al. 2011, 2012b, 2013; Rodríguez-Saltos et al. 2018). Neuromodulatory activity also varies on shorter time scales in response to social interaction or specific social signals in auditory, olfactory, and electrosensory systems (Hurley and Hall 2011; Matragrano et al. 2012a; Kabelik et al. 2014; Fotowat et al. 2016; Keesom and Hurley 2016; Fast and McGann 2017). This type of rapid neuromodulatory recruitment alters the selectivity of sensory neurons for different stimulus features, optimizes the encoding of stimuli in particular social contexts, or gates sensory activity (Hurley et al. 2004; Hurley and Pollak 2005; Devilbiss and Waterhouse 2011; Fast and McGann 2017; Tang and Trussell 2017; Marquez and Chacron 2018, 2020a, 2020b). These findings across taxa and sensory modality illustrate that sensory neuromodulators integrate information on context and state across multiple timescales and levels of organization (Forlano and Sisneros 2016; Hurley and Kalcounis-Rueppell 2018).

In the mouse IC, serotonin also responds to social interaction. Serotonin increases more in individuals showing greater social investigation and receiving less rejection (Hurley and Hall 2011; Keesom and Hurley 2016). Serotonergic neurons in two subregions of the DRN that project to the IC of females show opposite correlations with sociosexual behaviors, implying that serotonin is released from different DRN groups in different social conditions (Petersen et al. 2020). At the level of the responses of IC neurons, serotonin may change the selectivity of IC neurons for vocal signals, increase inhibition, or change response gain or timing (Hurley and Sullivan 2012). All of these findings suggest that serotonergic modulation of auditory processing depends on context and specific social contingencies. Isolation is likely to alter the balance of modulatory effects in the IC. Although the changes in responsiveness to serotonergic manipulation we report here were focused in the central IC subregion, previously reported increases in fiber density of females in social housing were most pronounced in the lateral IC subregion (Keesom et al. 2018). The central IC is

part of the ascending auditory pathway while the lateral subregion, although it receives substantial ascending auditory input, is also a site for multisensory integration (Malmierca 2004; Ito and Oliver 2012; Lesicko et al. 2016; Chen et al. 2018). Although the behavioral significance of serotonergic modulation for auditory processing is still unknown, our current findings complement a view of the serotonergic auditory regulation as highly responsive to behavioral context, social experience, and sex, and adds plasticity in postsynaptic responsiveness to serotonin as an important feature of this system.

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Conflict of interest

The authors declare that they have no conflict of interest.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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