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## Research report

# Social isolation reduces serotonergic fiber density in the inferior colliculus of female, but not male, mice

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## ABSTRACT

Early-life experiences, including maternal deprivation and social isolation during adolescence, have a profound influence on a range of adult social behaviors. Post-weaning social isolation in rodents influences behavior in part through the alteration of neuromodulatory systems, including the serotonergic system. Of significance to social behavior, the serotonergic system richly innervates brain areas involved in vocal communication, including the auditory system. However, the influence of isolation on serotonergic input to the auditory system remains underexplored. Here, we assess whether 4 weeks of postweaning individual housing alters serotonergic fiber density in the inferior colliculus (IC), an auditory midbrain nucleus in which serotonin alters auditory-evoked activity. Individually housed male and female mice were compared to conspecifics housed socially in groups of three. Serotonergic projections were subsequently visualized with an antibody to the serotonin transporter, which labels serotonergic fibers with relatively high selectivity. Fiber densities were estimated in the three major subregions of the IC using line-scan intensity analysis. Individually housed female mice showed a significantly reduced fiber density relative to socially housed females, which was accompanied by a lower body weight in individually housed females. In contrast, social isolation did not affect serotonergic fiber density in the IC of males. This finding suggests that sensitivity of the serotonergic system to social isolation is sexdependent, which could be due to a sex difference in the effect of isolation on psychosocial stress. Since serotonin availability depends on social context, this finding further suggests that social isolation can alter the acute social regulation of auditory processing.

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## 1. Introduction

Early-life social experience has a dramatic influence on a wide range of adult behaviors, including social behavior (Bibancos et al., 2007; Bolhuis et al., 2010; Bottjer and Arnold, 1997; Gariépy et al., 1995; Fone and Porkess, 2008; Kuramochi and Nakamura, 2009; Soga et al., 2015). Specifically, social interaction with parents and siblings during juvenile periods supports the normal development of a quintessentially social behavior: communication. In mammals and birds, early social experience influences vocal communication on both sides of the exchange: senders and receivers. As senders of signals, animals that are not exposed to species-typical vocalizations during early life display abnormal

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vocal production as adults (Bolhuis et al., 2010; Bottjer and Arnold, 1997; Prat et al., 2015; Takahashi et al., 2017). For example, young marmoset monkeys (0-2 months old) require vocal reinforcement from adults to demonstrate normal development of the adult-like contact-call (Takahashi et al., 2017). Similarly, male songbirds require experience with a tutor during juvenile periods to produce species-typical songs as adults (Bolhuis et al., 2010). Juvenile social experience also influences animals as receivers of vocal signals. At a behavioral level, an animal's response to particular adult vocalizations depends on its previous exposure to adult calls during early life (Asaba et al., 2014; Clayton, 1990; Vyas et al., 2009). For instance, adult female house mice (Mus musculus) prefer adult male vocalizations depending on the laboratory strain with which they were cross-fostered during post-natal days 0-21 (Asaba et al., 2014). Zebra finches demonstrate that behavioral discrimination between adult songs depends on early social environment, with cross-fostered females showing better discrimination among male songs of the subspecies with which they were raised, beginning 3–5 days post-hatching (Clayton, 1990). Finally, early





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Abbreviations: IC, inferior colliculus; SERT, serotonin transporter; 5-HT receptor, serotonin receptor.

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social experience also shapes receivers of vocal signals at a neural level. In songbirds, juvenile experience with adult conspecifics or their songs influences the selectivity, responsiveness, or information-coding capabilities of auditory neurons (Cousillas et al., 2004; Hahn et al., 2015; Hernandez and MacDougall-Shackleton, 2004; Woolley et al., 2010). Thus, social and auditory experiences in early life shape an animal's ability to receive and process vocal signals as an adult.

Plasticity in centralized neuromodulatory systems likely contributes to these experience-dependent changes in auditory reception. In particular, the serotonergic system is an excellent candidate mechanism for conveying integrated social information to the auditory system. With its cell bodies situated in the raphé nuclei, the serotonergic system receives a diversity of inputs from other brain regions involved in social processing (Pollak Dorocic et al., 2014: Vertes and Linley, 2008). Furthermore, serotonergic neurons in the raphé are activated by a variety of stimuli, including social partners (Li et al., 2016). This social information can then be distributed via the serotonergic system's widespread axonal network, which includes projections to multiple auditory nuclei (DeFelipe et al., 1991; Hurley and Thompson, 2001; Klepper and Herbert, 1991; Thompson et al., 1994). Upon its release, serotonin likely modulates the processing of vocal signals, because activation of serotonin receptors alters auditory-evoked activity in neurons across the auditory neuraxis (cochlear nucleus: Ebert and Ostwald, 1992; Felix et al., 2017; Thompson et al., 1994; medial superior olive: Ko et al., 2016; calyx of Held: Mizutani et al., 2006; periolivary regions: Wang and Robertson, 1997; inferior colliculus reviewed in Hurley and Sullivan, 2012; auditory cortex: Ji and Suga, 2007). This includes the mammalian auditory midbrain nucleus: the inferior colliculus.

The inferior colliculus (IC) of mice (Mus musculus) is a tractable system for exploring how serotonin conveys social experiential information to the auditory system, for several reasons. First, the IC is an important nexus in the auditory pathway. Auditory information converges in the IC, with ascending input from lower auditory brainstem nuclei, descending input from the auditory cortex, and input from contralateral structures (Casseday et al., 2002; Pannese et al., 2015). Thus, almost all acoustic information is processed by the IC. With particular relevance to communication, IC neurons demonstrate sensitivity to, and selectivity among, vocalizations (Holmstrom et al., 2007; Klug et al., 2002; Portfors et al., 2009; Xie et al., 2005). Furthermore, selectivity of IC auditory neurons is modulated by serotonin (Hurley and Sullivan, 2012), and serotonergic activity is induced by behavioral events, including interaction with conspecifics of both sexes (Hall et al., 2010; Hall et al., 2011; Hanson and Hurley, 2014; Keesom and Hurley, 2016). Serotonin release during social interaction also corresponds to social events in the recent past (Hall et al., 2011; Hanson and Hurley, 2014), as well as to long-term differences in social housing (Keesom et al., 2017a). Specifically, serotonin increases more quickly and reaches its maximal amplitude sooner in socially housed mice compared to individually housed mice. Experiencedependent plasticity in serotonergic fiber density in the IC could contribute to this effect because social experience influences the projection fields of serotonergic neurons to other brain regions (Kuramochi and Nakamura, 2009; Lehmann et al., 2003; Poeggel et al., 2003; Soga et al., 2015; Whitaker-Azmitia et al., 2000). Although the influence of social experience on serotonergic fiber density in the IC is unknown, other types of experience have been studied. For example, prolonged exposure to high amplitude sound (acoustic trauma) alters the density of serotonergic fibers in adults, demonstrating plasticity in serotonergic projections to the IC (Papesh and Hurley, 2012).

To assess whether social experience influences serotonergic fiber density in the IC, we compared the density of fibers labeled with an antibody to the serotonin transporter (SERT) in CBA/I mice that were housed individually or in social groups of three for a period of 4 weeks post-weaning. This is a simple manipulation with powerful effects both on social behaviors (Bibancos et al., 2007; Keesom et al., 2017b; Kuramochi and Nakamura, 2009; Soga et al., 2015) and on the serotonergic system in brain areas outside the auditory system, including synthetic enzyme activity, serotonergic receptor expression, serotonin release and reuptake, and fiber density (Ago et al., 2013; Bibancos et al., 2007; Dankoski et al., 2014; Fone and Porkess, 2008; Fulford and Marsden, 1998; Jones et al., 1992; Lukkes et al., 2009; Muchimapura et al., 2002; Schiller et al., 2003; Schiller et al., 2006; Segal et al., 1973). We further compared fiber densities in males and females, because the effects of social housing on the serotonergic system may be sexspecific (Ago et al., 2013). Based on the effects of social experience in other brain regions (Lehmann and Lehmann, 2007; Kuramochi and Nakamura, 2009: Poeggel et al., 2003: Whitaker-Azmitia et al., 2000), we predicted that post-weaning social isolation would decrease the density of serotonergic fibers within the IC.

#### 2. Results

Multiple factors influenced the densities of SERTimmunoreactive fibers in the IC, which were estimated by measurement of fibers crossing a grid superimposed on the IC. Fiber densities of males and females were initially analyzed in separate statistical models Revi because of the need for different sets of cofactors for each sex (see Experimental procedure section). With these models, fiber density depended not only on the IC subregion, but also on whether mice were housed individually or in social groups, in a sex-specific manner. Although fiber density also varied with cohort (mice that experienced the housing treatment at the same time), cohorts were balanced to include an equal representation of individually and socially housed mice.

## 2.1. SERT fiber density varies across inferior colliculus subregion

Subregions of the IC were defined by an antibody to nitric oxide synthase (Fig. 1A; Coote and Rees, 2008). In both male (F (2,15) = 128.4, p < 0.001) and female (F (2,13) = 34.14, p = 0.001) mice, the densities of SERT-immunoreactive fibers were different across the three subregions of the IC (Fig. 2A). For males and females in both social treatment conditions, SERT-immunoreactive fiber density was lower in the central nucleus (CNIC) than in the lateral cortex (LC) and dorsal cortex (DC) (Fig. 2B and C). This result is consistent with a previous study using a stereological approach to estimating fiber density (Papesh and Hurley, 2012).

## 2.2. Sex difference in the effects of social isolation

Individually and socially housed male mice showed no difference in either the overall densities of serotonergic fibers in the IC (F (2,15) = 1.64, p = 0.23), or in their distribution across different IC subregions (F (2,15) = 0.47, p = 0.95). This is depicted in Fig. 2B, in which individually housed males (black bars) demonstrate fiber densities that are indistinguishable from those of socially housed males (white bars) in each IC subregion. In contrast, female mice showed differences in overall fiber densities depending on whether they were housed individually or socially (F (2,13) = 19.25, p = 0.01), and in the distribution of fibers across IC subregions (F (2,13) = 17.09, p < 0.001). Fig. 2C illustrates the significant main effect of social treatment on fiber density, with socially housed females having a higher density of SERTimmunoreactive fibers. While the DC and LC show indistinguishable fiber densities in individually housed females, the LC has a



Fig. 1. (a) An antibody to neuronal nitric oxide synthase (nNOS) was used to identify subregions of the IC. (b) SERT-immunoreactive fibers. (c) Schematic image of grid imposed on immunohistochemical images for the purpose of fiber density estimation.



**Fig. 2.** (a) Representative depictions of SERT-positive fibers in the three subregions of the IC. (b) Mean fiber densities in the three IC subregions of males that were housed individually (IND: black bars, n = 9) versus socially (SOC: white bars, n = 9). The CNIC has significantly fewer fibers than either the LC or DC in both housing treatments. (c) Mean fiber densities in the three IC subregions of females that were housed individually (IND: black bars, n = 8) versus socially (SOC: white bars, n = 8). Fiber densities are lowest in the CNIC for each housing group. Additionally, in the socially housed females, fiber densities are higher than in the individually housed females, and the distribution of fibers among subregions is different than for the individually housed females.

higher fiber density than either the CNIC or DC in socially housed females. Whether females were in a receptive phase of the estrous cycle (estrus and proestrus) or a nonreceptive phase (metestrus and diestrus) had no impact on serotonergic fiber density (F (2,13) = 0.45, p = 0.54).

We further explored the effects of cohort on fiber density. A "cohort" of six same-sex mice began the housing treatment at the same time. Social treatments were balanced within each cohort, which had equal numbers of socially housed (n = 3) and individually housed (n = 3) mice per cohort. There were three cohorts per sex. There was a significant effect of cohort on fiber density in the IC (males: F(2,15) = 81.15, p < 0.001; females: F(2,13) = 8.84, p = 0.03). Within both males and females, one of the cohorts had different fiber density levels than the others, most notably in the LC

(Fig. 3). For males, fiber density was higher in one cohort than in the other two cohorts. For females, fiber density in one cohort was lower than in the other two cohorts. We cannot positively attribute these cohort differences to any specific factor (see Discussion). Although each of the cohorts in Fig. 3 showed qualitatively similar effects of the social treatment, the variability introduced by the third cohort made it difficult to compare sexes. We therefore directly compared male and female fiber densities using the two consistent cohorts for each sex (M1 and M2 versus F1 and F2). A repeated measures ANOVA with IC subregion as a within-subjects factor and with social treatment and sex as between-subjects factors revealed significant effects of region and sex (region: F (2,20) = 80.85, p = <0.001; sex: F (2,20) = 18.28, p < 0.001). There was no main effect of social treatment across sexes, but there was an interaction between sex



**Fig. 3.** Fiber densities of the LC in different cohorts (M1-M3 and F1-F3). Open circles represent socially housed mice (SOC), and filled circles represent individually housed mice (IND). Individually housed and socially housed mice in the same cohort are slightly offset from each other for better comparison.

and social treatment (F (2,20) = 4.61, p = 0.045). Posthoc tests (Fisher LSD) revealed no sex difference in the individually housed mice for any IC subregion (LC: p = 0.25; DC: p = 0.26; CNIC: p = 0.10). For the socially housed mice, there were significant sex differences in all regions (LC: p < 0.001; CNIC: p = 0.001; DC: p = .014). Using the consistent cohorts, males and females therefore had similar fiber density when housed individually, but diverged in fiber density in same-sex social housing.

In conjunction with our finding of a sex difference in the effect of housing condition on serotonergic fiber density, we also found a difference in the effect of housing condition on animals' body weights. Body weight provides insight into an animal's bioenergetic status, and previous studies demonstrate that body weight is influenced by both neuroendocrine factors and psychosocial stress, including social isolation (Ross et al., 2017; Saegusa et al., 2011). In our study, female mice that were housed individually weighed significantly less than those housed socially whereas males showed no difference in weight between treatment groups (Fig. 4; n = 6 for each female group and n = 9 for each male group). The difference in average weight of 1.9 g between groups in females amounted to 9.9% of the average body weight of 19.1 g of females in the study.

## 3. Discussion

Post-weaning isolation induced a modest but significant decrease in serotonergic fiber density relative to standard social



**Fig. 4.** Weights of males and females in different housing treatments were measured, because previous studies indicate that psychosocial stress, such as social isolation, affects body weight (Ross et al., 2017; Saegusa et al., 2011). Weights of males (n = 9 per treatment) did not differ individually and socially housed animals, but weights of individually housed females were lower than weights of females housed in social groups (n = 6 per treatment). Note that y-axis begins at a non-zero value.

housing in the IC of CBA/J mice. This effect occurred in females but not in males, and was associated with a significantly lower body weight in isolated females, but not males. When subsets of males and females were directly compared, males and females had the same fiber densities when in individual housing, but differed when in same-sex social housing. The current study also replicated a past quantitative observation that serotonergic fiber density is higher in the LC and DC than in the CNIC (Papesh and Hurley, 2012).

These findings are consistent with a substantial literature demonstrating that post-weaning isolation alters the anatomy and function of serotonergic pathways (e.g., Ago et al., 2013; Bibancos et al., 2007: Fone and Porkess, 2008: Fulford and Marsden, 1998; Schiller et al., 2006; Segal et al., 1973). In terms of serotonergic projections, social isolation either increases or decreases serotonergic fiber density in a wide range of brain regions. These regions include the basolateral and central nuclei of the amygdala (increase: Lehmann et al., 2003; decrease: Kuramochi and Nakamura, 2009), the CA3 region of the hippocampus (decrease: Kuramochi and Nakamura, 2009; Whitaker-Azmitia et al., 2000), and the prefrontal and infralimbic cortices (increase: Braun et al., 2000; Poeggel et al., 2003). The effects of isolation on behavior and serotonergic pathways are also frequently sexdependent (Ago et al., 2013; Fone and Porkess, 2008; Schiller et al., 2006).

Serotonin is an important modulator of the central auditory system (Felix et al., 2017; Ko et al., 2016; Mizutani et al., 2006; Tang and Trussell, 2017), that reflects social and stressful contexts in the IC (Hall et al., 2011; Hall et al., 2012). Our findings suggest that social isolation could therefore contribute to the dysregulation of auditory activity in these contexts. Below, we describe potential cues for plasticity in auditory-serotonin interactions, consider factors that were significantly associated with differences in SERT fiber density, and address the potential consequences of altered fiber density for serotonergic and auditory function.

## 3.1. Cues mediating plasticity in auditory-serotonin interactions

Multiple types of stimuli associated with social interaction could have diverged for our socially and individually housed mice, potentially accounting for differences in serotonergic fiber density. Sensory cues such as tactile aspects of social contact, odor, and the acoustic environment could all have been richer for socially housed animals (Arakawa et al., 2008; Woolley, 2012). The serotonergic system is highly responsive to a range of social stimuli (Kiser et al., 2012), and conditions such as social isolation have wide-spread effects on serotonergic function in the brain (reviewed in Fone and Porkess, 2008). An effect of social cues that was not specific to any particular sensory modality is therefore a possible mechanism for the effects we observed.

Because the IC is a primary auditory region, the influence of the social housing treatment on serotonergic fiber density could also have been mediated by social vocalizations in particular. Although we did not measure vocalizations over the month of social treatment, individually housed animals could not have been exposed to vocalizations from cage mates. There is thus an intrinsic difference in acoustic environments between social and individual housing. This difference is interesting to consider in light of past studies showing that deprivation of acoustic stimulation due to induced hearing loss influences serotonergic fiber density as well as the expression of serotonin receptors in the IC (Papesh and Hurley, 2012; Smith et al., 2014). Calling between pairs of female mice is greatest when they are first placed together, and declines substantially as they gain familiarity (Hoier et al. 2016). This decline argues against a purely acoustic effect of social housing. However, since vocalizations have great social salience, it is possible that they could have had an effect on serotonergic fibers, even if vocalizations were rare.

#### 3.2. Variation between sexes

In this study, we demonstrate a sex-specific effect of social experience on serotonergic fiber density, with social isolation decreasing fiber density in the IC of females, but not males. This sex-specific effect is an interesting finding, considering the natural ecology of house mice. Generally, free-living populations of house mice exhibit a social structure whereby a dominant male mouse occupies a territory in which a few subordinate males and several females reside (Berry & Bronson, 1992; Bronson, 1979; Chambers et al., 2000; Gray et al., 2000). Males exhibit a stable social hierarchy that is determined by aggressive contests, whereas social status is determined by intrinsic characteristics for females (Van den Berg, 2015). These differing mechanisms are reflected in the increased intrasexual agonistic behavior displayed by males, whereas females show increased affiliative behavior in groups (Arakawa, 2018; Palanza et al., 1993; Parmigiani et al., 1999). Individual housing and social housing may therefore be differentially "stressful" for mice of different sexes. This idea is in part supported by our finding of decreased body weight of female mice in individual housing compared to social housing, but no housing effect on male body weight. This coincides with previous studies that have demonstrated decreased weights in socially isolated females, but not males (Ross et al., 2017; Saegusa et al., 2011). Physiological factors that regulate energy balance, such as corticosteroids, likely contributed to our observed divergence in social effects on body weight. For example, individually housed female rodents display elevated corticosteroids compared to group-housed females (Benite-Ribeiro et al., 2014), whereas the opposite is found in males (Brown and Grunberg, 1995). Behavioral studies also suggest that individual housing is more "stressful" for females. Female rodents show increased anxiety-related behaviors after individual housing compared to group-housing, whereas males show decreased anxiety after individual housing (Palanza et al., 2001). Thus, the sex-specific effects of social housing we report may be due to increased psychosocial stress experienced by the individually housed females relative to the socially housed females, with either a direct influence on the serotonergic system (which is itself sensitive to social factors) or an indirect effect via metamodulation by other neuroendocrine systems.

Neuroendocrinological milieu is another significant way in which female and male mice differ, especially as female reproductive hormones fluctuate through the four-day estrous cycle. In the current study, the estrous phases of female mice did not correspond to serotonergic fiber density. These findings suggest that estrous phase does not strongly influence fiber density in the IC of female mice, and provide an interesting comparison to animals with reproductive phases that cycle on a longer time scale. In white-throated sparrows, which are seasonally breeding birds, the administration of estradiol increases serotonergic fiber density in auditory midbrain and forebrain regions, and increases the concentration of a serotonergic metabolite in auditory forebrain (Matragrano et al., 2012). The finding that estrous phase does not strongly influence serotonergic fiber density in mice does not mean that estrous phase has no influence on the serotonergic system at other levels, however. In mice, estrous phase influences the numbers of c-Fos immunoreactive neurons in the IC following the direct manipulation of serotonin levels (Hanson and Hurley, 2016).

#### 3.3. Variation among IC subregions

Of the three distinct subregions of the IC, the LC showed the most pronounced effect of social treatment. The topography of raphe projections to the different subregions of the IC has not been characterized, so it is unclear whether the LC is targeted by a distinct set of raphe neurons that has greater sensitivity to the social environment. It is also possible that intrinsic distinctions among IC subdivisions result in differential social sensitivity. The LC is functionally and anatomically quite distinct from the CNIC. The CNIC is the main recipient of auditory information ascending from a range of auditory brainstem nuclei, and a major principle of its organization is a tonotopy that reflects the layout of the cochlea (Loftus et al., 2008; Malmierca et al., 2008). The LC receives substantial feedback from auditory cortex and subcortical sites as well as tonotopically organized projections from the cochlear nucleus (Loftus et al., 2008: Stebbings et al., 2014: Patel et al., 2017). The LC also receives inputs from non-auditory sensory modalities, and a prominent organizational feature of this region is a series of neurochemical modules related to auditory versus somatosensory processing (Zhou and Shore, 2006; Lesicko et al., 2016). Whatever the cause of the differential sensitivity of serotonergic fibers among IC subregions, our findings suggest that the serotonergic milieu of the LC would be most strongly influenced by social experience in female mice.

#### 3.4. Variation among cohorts

In both male and female groups, a single cohort was different in overall fiber density from the other two cohorts, although the direction of the difference varied depending on sex. To assess whether any experimental condition related to these third cohorts, we assessed a range of factors including housing conditions, personnel, immunohistochemical reagents, and time of year. Of all of these factors, only the time of year in which the mice were shipped and tested related exclusively to both the male and female third cohorts. The third cohorts were each shipped during winter months (December and January), while the other cohorts were shipped in late spring/early summer (late April through June). An effect of stress due to colder temperature exposure while shipping, or a developmental effect of photoperiod on the serotonergic system, are certainly possibilities that could account for our results. Such effects could be mediated through the developmental sensitivity of the raphe system to both stress and photoperiod, which in some cases is sex-dependent (Green et al., 2015; Lukkes et al.,

2016). However, we exercise caution in concluding that the shipping season was causal to the discrepancies we observed among cohorts. This is both because we have no direct evidence of this relationship, and because it is possible that additional circumstances beyond our knowledge contributed to the inter-cohort differences.

#### 3.5. Consequences of increased fiber density

Several recent studies on post-weaning social isolation in mice suggest that isolation is associated with an inability to appropriately calibrate social behavior and serotonin release in the IC with the current social environment (Keesom et al. 2017a,b). Pairs of unfamiliar individually housed male mice produce ultrasonic vocalizations at more than double the rate of pairs of unfamiliar socially housed mice (Keesom et al., 2017b). These vocal changes correspond to greatly increased rates of social investigation. Interestingly, rates of mounting behavior, sometimes interpreted as a sign of aggression between males, is similarly low between social-social and isolated-isolated pairs, but is high in pairs consisting of one socially housed and one individually housed male (Keesom et al., 2017b). These patterns may indicate that previously isolated mice lack the ability to regulate their behavior according to context, and escalate their aggressive nonvocal and vocal behavior in an otherwise nonthreatening interaction. Serotonergic activity in the IC of individually housed males interacting with other males similarly shows a lack of correlation with social behavior, whereas serotonergic activity is positively correlated with social interaction in socially housed males (Keesom et al., 2017a). Although these previous findings suggest a differential regulation of serotonin in the IC depending on social experience, our current findings suggest that this difference is not attributable to serotonergic fiber density, since males showed no effect of social experience on fiber density (Fig. 2B).

Our interpretation of the consequences of increased serotonergic fiber density in socially housed females must also consider that serotonergic fibers were labeled with an antibody to SERT. This molecule labels serotonergic projections relatively selectively and stably (Fujita et al., 1993), but also regulates serotonin availability following its release. Pharmacologically reducing SERT function increases the amplitude of serotonergic transients following stimulated release, and also greatly prolongs these transients (Hashemi et al., 2012). The fact that socially housed females had a higher density of SERT-labeled fibers therefore suggests several possibilities. A higher density of projections from raphe nuclei could be associated with greater serotonin release in situations associated with increased serotonin in females, including during social interactions (Hanson and Hurley, 2014). Alternatively, the dynamics of serotonin availability could also be more tightly regulated in socially housed females with a higher density of SERT-bearing fibers.

### 3.6. Social isolation and sensory processing

Our current findings suggest that the effects of social isolation encompass auditory regions like the IC. Studies on the effect of serotonin on the activity of IC neurons provide a background for understanding the potential outcomes of the serotonergic changes triggered by housing conditions. Although the behavioral outcomes of serotonergic modulation in the IC are not yet wellunderstood, serotonin makes the responses of IC neurons to vocalizations more selective in general (Hurley and Pollak, 2005), and preferentially decreases the activation of IC neurons during nonsocial contexts relative to social contexts (Hanson and Hurley, 2016). Additionally, depletion of the serotonergic system with pharmacological agents, as opposed to isolation, alters the auditory brainstem response (ABR) by decreasing the latencies of waves II through V in a frequency-dependent manner (Papesh and Hurley, 2016). Serotonergic activity in the IC is sensitive to context in that it generally increases during social interaction, but also correlates with the social behaviors displayed by experimental subjects as well as by their social partners (Hall et al., 2011; Hanson and Hurley, 2014; Keesom and Hurley, 2016). Our current findings therefore raise the possibility that individual housing could alter the sensitivity of auditory processing within the IC to context.

## 4. Experimental procedure

## 4.1. Animals

The effect of social isolation on serotonergic fiber density within the inferior colliculus (IC) was examined using eighteen female and eighteen male CBA/J mice (Mus musculus; The Jackson Laboratory, Bar Harbor, ME). A total of nine mice of each sex were used for each treatment (individual versus social housing). However, the final sample sizes for females were eight individually housed mice and eight socially housed females due to insufficient fixation of brain tissue in two animals. Cohorts of six non-kin, same-sex mice (post-weaning,  $\sim 21$  days old) were ordered from the supplier. Upon arrival, three mice from each cohort were placed in cages alone ("individual housing") and the remaining three mice were housed as a same-sex group in a single cage ("social housing"). Mice were maintained in institutional animal guarters on a 12-h light cycle, fed standard mouse chow, and provided water ad libitum. All procedures were approved by the Institutional Animal Care and Use Committee at Indiana University, Bloomington.

## 4.2. Determining estrous stage

For four consecutive days prior to beginning histology, female mice were assessed for estrous stage via vaginal cytology. Vaginal cells were collected via lavage, and the matrix of saline and vaginal cells was dispensed onto a glass slide. After drying, the slides were submerged in methanol for 10 min, followed by 15 min in Giemsa stain. Estrous phase was determined by the presence of different vaginal cell types, as viewed under a 20X bright field microscope: nucleated epithelial cells indicated proestrus, cornified epithelial cells indicated diestrus (Byers et al., 2012; Finton et al., 2017; Goldman et al., 2007).

#### 4.3. Social dominance test

Plasticity in the serotonergic system has been associated with dominance status across different species (Chiao, 2010; Watanabe and Yamamoto, 2015). Therefore, at the conclusion of the four-week housing period, group-housed mice were tested to determine the social hierarchy using a tube dominance test. This assay provides a reliable measure of dominance corresponding to multiple physiological variables (Lindzey et al., 1961; Howerton et al., 2008; Wang et al., 2011). The testing apparatus consisted of a transparent tube connecting two enclosed chambers of a standard laboratory cage. The tube, dividers, and cage were cleaned with laboratory detergent and wiped with 70% ethanol between each trial. Same-sex mice were placed on opposite sides of the transparent tube and released simultaneously. Behavior was video-recorded during each 5-min trial. When both mice were inside of the tube, the subordinate mouse would move backwards while the dominant mouse continued moving to the opposite side. Mice were considered dominant if they occupied the tube for the duration of the trial, and subordinate if they occupied one of the side chambers for the duration for trial. Using a round-robin tournament design, a pattern of dominance emerged of one dominant and two subordinate mice per group in the socially housed males, as previously reported (Wang et al., 2011). Dominance status did not correspond to serotonergic fiber density (F (2,6) = 0.024, p = 0.88), but the sample size was low (n = 3 dominant males and n = 6 subordinate males). Socially housed female mice were tested for dominance, but did not show reliable dominance hierarchies.

## 4.4. Immunohistochemistry

At the conclusion of the tube dominance assay, mice were weighed and subsequently perfused with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) at pH 7.3. The brains were extracted and placed into a 15% sucrose, ~4% paraformaldehyde PBS solution for 24 h. This was followed by placing the brains in 30% sucrose PBS solution for an additional 24 h. A sliding microtome was used to acquire 50- $\mu$ m coronal tissue sections of the IC and surrounding areas. Sections were collected as four adjacent series: one series was placed into PBS (for SERT immunohistochemistry) and three series were placed into cryoprotectant (one series for nNOS immunohistochemistry), which were then kept frozen at -80 °C until later use.

The serotonin transporter (SERT) was immunofluorescently labeled in order to assess serotonergic fiber densities. Targeting SERT reveals patterns and densities of serotonergic axons comparable to labeling serotonin itself, with decreased nonspecific background staining (Mamounas et al. 2000; Nielsen et al., 2006). Tissue sections from each cohort of six same-sex mice (three individually housed, three socially housed) was processed simultaneously for all immunohistochemistry. Free-floating tissue sections were first rinsed 4 times in PBS, after which tissue sections were placed in a general blocking solution (10% normal donkey serum in a solution of 0.3% Triton-X and 0.1 M PBS, pH 7.3) for one hour. Tissue sections were then incubated in primary rabbit anti-SERT antibody (1:2000, Immunostar, Catalog #24330) for 36 h at 4 °C. After incubation, tissue sections underwent two 15-min rinses in PBS. Sections were then incubated with a secondary donkey antirabbit antibody conjugated to Alexa Fluor 488 (3:1000, Invitrogen, Catalog #A-21206) for 2 h in the dark, followed by two rinses in PBS. Tissue sections were then mounted on chromium gel-coated slides and cover-slipped with ProLong Gold anti-fade mountant (Life Technologies, Catalog #P36930) and stored in a dark location. Excluding primary antibody and pre-adsorbing the primary antibody with a control peptide both resulted in a lack of SERT fiber labeling (Fig. 5A-C), similar to a past study (Papesh and Hurley, 2012).

The distribution of neuronal nitric oxide synthase (nNOS) immunoreactive cells varies by subregion (Coote and Reese

2008). We therefore used nNOS to delineate the architecture of the IC (Fig. 1). The DC and LC were identified by elevated levels of nNOS immunoreactivity compared with the CNIC; Coote and Rees, 2008; Loftus et al., 2008). nNOS tissue sections were labeled using the same methodology as for SERT with the substitution of the primary rabbit anti-nNOS antibody (1:2000, Immunostar, Catalog #24287) for the primary rabbit anti-SERT antibody. Personnel conducting imaging and analysis were blind to treatment group.

## 4.5. Fluorescent microscopy and image processing

Fluorescent microphotographs were taken using an Applied Precision DeltaVision personalDV microscope (GE Healthcare Life Sciences). For each SERT-immunoreactive tissue section, a mosaic of 20X images ( $1024 \times 1024$  pixels per image,  $0.321 \,\mu$ m/pixel) allowed capture of the entire inferior colliculus across multiple zplanes (25–30 per tissue section, 1.3 µm thickness) using an automated stage, and the resulting images were stitched together into one image by softWoRx software (GE Healthcare). All images of SERT-immunoreactive tissue were captured using the same light and camera settings across subjects. The resulting images were calibrated and deconvolved, and z-planes were collapsed into one maximum projection image per tissue section using softWoRx. nNOS-immunoreactive tissue sections were captured at 4X magnification (1024  $\times$  1024 pixels, 1.605  $\mu$ m/pixel). All photomicrographs of nNOS-immunoreactive tissue were captured using the same light and camera settings across subjects (10% transmission of light, 0.300 ms exposure time).

Additional processing of images was done in NIH ImageJ (version 2.0.0). All images were converted to 8-bit grayscale. This was followed by applying a Hessian filter (FeatureJ plugin) (FeatureJ plugin; Meijering et al., 2004), which enhances curvilinear structures, such as fluorescently labeled fibers. Subsequently, the outline of the IC and its subregions were drawn on the SERT-immunoreactive images by referencing the images of adjacent tissue sections immunofluorescently labeled for nNOS (Fig. 1AB). Each subject animal was represented by two tissue sections separated by at least 150  $\mu$ m within a range of 4.84–5.20 mm caudal to Bregma including the right and left IC (Paxinos and Franklin, 2004).

## 4.6. Fiber density measurements

Line-scan intensity analysis, adapted from Sathyanesan et al. (2012), was used to estimate fiber density. In this technique, a plot of pixel gray-values along a line ("line-scan") drawn on an image of fluorescently labeled tissue is generated, where peaks in pixel intensity represent fluorescent fibers that intersect the line. Line-scans were collected every 100  $\mu$ m in ImageJ using the "line tool",



**Fig. 5.** (a) SERT-immunoreactive fibers. (b) Section processed with no SERT-antibody. (c) Section processed with SERT antibody that had been preadsorbed with the control peptide before incubation of the section. All sections were alternate slices from the same individual, and were immunohistochemically processed in parallel. Photomicrographs were taken in the LC.

and the X and Y values from the pixel intensity plots were imported into MatLab (R2015a; The MathWorks, Inc.). A custom script in Matlab was used to count fibers, incorporating MatLab's Bioinformatics toolbox command "msbackadj" to estimate the true baseline of the scans and the "mspeaks" command to identify local maxima above a specified threshold for each image, interpreted as locations where a fluorescent fiber interested the line-scan, as in Sathyanesan et al. (2012).

For each tissue section, we collected two series of line-scans, one in the dorsoventral plane and one in the mediolateral plane. Line-scans of both series were collected every 100  $\mu$ m, beginning 50  $\mu$ m from the lateral extent (for dorsoventral line-scans) and 50  $\mu$ m from the dorsal extent (for mediolateral line-scans) of the IC. In an error analysis in which we conducted five trials, with line-scans slightly off-set each time, this 100- $\mu$ m sampling frequency had low error among the number of fibers counted in the 5 trials (<5% error). Line-scans were collected separately by subregion to allow for fiber density estimation by subregion, but were concatenated during some analyses.

Background threshold was determined by collecting gray values for 6-µm samples in the interstitial space between visually identified fibers. These gray values were imported into a custom MATLAB script that utilized the function "msbackadj". Once a background threshold was determined, the average rate of fiber intersection was calculated for both mediolateral and dorsoventral line-scans by dividing the total number of fiber-related intersections by the line's length (µm). A fiber density index was calculated by multiplying the mediolateral rate of fiber intersections by the dorsoventral rate of fiber intersections. This value was then divided by the total thickness of the z-planes in the projected image  $(1.3 \,\mu m/z)$ plane) and multiplied by the thickness of the tissue sections (50 μm) in order to obtain a corrected three-dimensional estimate of fiber density. Final fiber densities for a given animal were calculated by averaging the corrected fiber densities of two representative tissue sections per animal.

We tested the reliability of our method for consistently counting fibers, both in different regions on the same section from the same animal (using the same threshold) and on sections from different animals (using thresholds calculated for each sample). For the "within-animal" test, an observer manually counted fibers along twelve different line-scans distributed equally across the tissue; this was done for two images, each from a different animal. For the "between-animals" test, an observer counted fibers along one line-scan on ten different images from ten different animals. All manual counting of fibers intersecting line-scans were conducted by a single observer. Matlab-counted fibers were highly and significantly correlated with manually counted fibers, both within animals (r = 0.971, df = 10, p < 0.001) and across different animals (r = 0.953; df = 8, p < 0.001).

#### 4.7. Statistical analysis

SERT-immunoreactive fiber densities were assessed in Statistica using repeated-measures ANOVAs with the subregion of the IC (CNIC, DC, LC) as a within-subjects factor and social treatment and cohort as between-subjects factors. Posthoc comparisons were made with the Fisher least significant difference (LSD). Fiber densities from males and females were considered in separate statistical models. This was because males and females had different additional cofactors in that females, but not males, could be in different phases of estrous. Another difference between sexes was that males, but not females, showed definitive dominant or subordinate behaviors in the tube test. Because dominant/submissive status was relevant for socially housed but not singly housed males, this was tested in a separate repeated measures ANOVA with IC subregion as a within-subjects factor and dominance status as a between-subjects factor. Dominance status did not correspond to serotonergic fiber density (F (2,6) = 0.024, p = 0.88), but the sample size was low (n = 3 for dominant males and n = 6 for subordinate males). A subset of males and females were also directly compared in a repeated measures ANOVA with IC subregion as a within-subjects factor and social treatment and sex as between-subjects factors. Weights of mice in the socially versus individually housed groups were compared using t-tests.

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