Multiple Postsynaptic Protein Levels in Adult Superior Colliculus Are Unaffected by Dark Rearing from Birth

Abbreviated title: Postsynaptic proteins stable in dark rearing

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Abstract

Visual deprivation by dark rearing in kittens and monkeys delays visual pathway development and prolongs the critical period. In contrast, receptive fields (RFs) in superior colliculus (SC) of Syrian hamsters *(Mesocricetus auratus)* refine normally with spontaneous activity alone, requiring only brief juvenile visual experience to maintain refined RFs in adulthood (Carrasco et al., 2005). Extending dark rearing past puberty leads to lower GAD and GABA levels due to reduced BDNF-TrkB signaling, resulting in RF re-enlargement (Carrasco et al., 2011; Mudd et al., 2019). Previous studies in kittens and monkeys have reported that dark rearing is associated with changes in both GABA ligand and $GABA_A$ receptor levels. Given the reduced GABA levels in SC of dark reared adult hamsters, we asked if dark rearing also causes changes in $GABA_A$ receptor levels. We examined expression of $GABA_A$ receptor subunits, their anchoring protein gephyrin, and the cation-chloride co-transporters KCC2 and NKCC1 in dark reared hamsters. Surprisingly, we found that dark rearing from birth until puberty had no effect on the levels of any of these postsynaptic elements, revealing a new form of maladaptive, presynaptic only inhibitory plasticity in which, rather than extending the critical period as seen in kittens and monkeys, hamster receptive fields refine normally and then lose refinement in adulthood. These results suggest that attempts to increase plasticity in adulthood for rehabilitation or recovery from injury should consider the possibility of unintended negative consequences. In addition, our results demonstrate the interesting finding that changes in neurotransmitter levels are not necessarily coordinated with changes in postsynaptic components.

72 # Introduction

73 During brain development, synaptic connections are elaborated and then refined to a mature state under
74 the influence of neural activity. Activity due to sensory experience during an early "critical period" is
75 important for shaping some aspects of neural circuit development. In the visual pathway, although
76 spontaneous retinal activity is important for initial axon pruning (Kutsarova et al., 2017), it has long been
77 thought that early visual experience is essential to attain refined connectivity patterns during development
78 (Wiesel and Hubel, 1965; Maffei and Galli-Resta, 1990; Meister et al., 1991; Wong et al., 1993; Katz and
79 Shatz, 1996; Firth et al., 2005). Once critical periods have closed, plasticity is often limited or even
80 prevented, thus protecting refined circuits from destabilization (Hubel and Wiesel, 1970; Takesian and
81 Hensch, 2013; Hübener and Bonhoeffer, 2014; Pallas, 2017; Hensch and Quinlan, 2018; Reh et al., 2020;
82 Ribic, 2020; Mitchell and Maurer, 2022).

83 In some mammals, dark rearing is reported to delay or prevent refinement, prolonging critical period
84 plasticity (Cynader and Mitchell, 1980; Mower et al., 1985; Mower, 1991; Lee and Nedivi, 2002;
85 Nakadate et al., 2012). In apparent contradiction to this common view, we have reported that spontaneous
86 activity is sufficient for refinement of receptive fields in both visual cortex (V1) and superior colliculus
87 (SC) of dark reared (DR) Syrian hamsters *(Mesocricetus auratus)*, and early light exposure for 3-7 days is
88 necessary only to maintain the refinement into adulthood (Carrasco et al., 2005; Carrasco and Pallas,
89 2006; Balmer and Pallas, 2015; Mudd et al., 2019). Syrian hamster pups spend the first 3-4 weeks after
90 birth underground in the wild (Adler, 1948; Nowosielski-Slepowron and Park, 1987), so it would not be
91 beneficial to have the development of their visual function depend on light exposure. If early visual
92 experience continues to be unavailable, GABAergic lateral inhibition in SC and V1 declines and RFs
93 expand, but not until approximately two months of age (~puberty) (Carrasco et al., 2005; Balmer and
94 Pallas, 2015; Mudd et al., 2019). Pharmacological activation of TrkB receptors can mimic the effects of
95 early light exposure in DR hamsters, resulting in long-term maintenance of refined receptive fields and
96 visual acuity (Mudd et al., 2019), perhaps through promoting GABA synthesis (Zhang et al., 2018).

97 Thus, hamsters, contrary to what has been found in cats and monkeys, need visual experience to maintain
98 refined receptive fields in adulthood, but not to refine them during development **(Figure 1)**.
99 $GABA_A$ receptors are pentameric, ionotrophic receptors consisting of five subunits grouped around a
100 central chloride ion pore. The functional characteristics of the receptor largely depend upon the subunit
101 composition (Sigel et al., 1990) and organization (Minier and Sigel, 2004). Of the many subunit
102 arrangements, alpha1 and alpha2 subunits have been linked to synaptic localization of $GABA_A$ receptors.
103 However, these two subunit types have different kinetics and are expressed at different points in
104 development. At birth, receptors containing the alpha2 subunit are widely expressed throughout the brain,
105 whereas alpha1 expression is initially low in major areas of the brain like the neocortex, the hippocampus,
106 and the cerebellum (Laurie et al., 1992; Fritschy et al., 1994; Dunning et al., 1999; Chen et al., 2001).
107 During the first several postnatal weeks, alpha1 expression increases, coinciding with a reduction in
108 alpha2 expression (Fritschy et al., 1994). This alpha2 to alpha1 switch in subunit expression underlies a
109 developmental decrease in inhibitory postsynaptic current (ipsc) decay time and an increase in ipsc
110 amplitude (Fritschy et al., 1994; Okada et al., 2000; Yu et al., 2006).
111 Our lab demonstrated previously that the expansion of RFs in SC of adult, DR hamsters is associated
112 with a loss of GABA immunoreactivity (Carrasco et al., 2011). Iontophoretic application of $GABA_A$
113 agonists *in vivo* restored RFs to a normal adult size. In addition to a loss of GABA-immunoreactivity,
114 $GABA_A$ agonists and antagonists were less effective in SC and V1 neurons of DR hamsters than in
115 normally reared (NR) hamsters (Carrasco et al., 2011).
116 The incomplete development of RF properties in V1 of visually deprived cats has been associated
117 with a failure in developmental maturation of NMDA and $GABA_A$ receptors (Carmignoto and Vicini,
118 1992; Chen et al., 2000; Chen et al., 2001; Erisir and Harris, 2003). Although a failure to maintain refined
119 RFs is a different phenomenon than a failure to refine them during a critical period, the mechanism(s)
120 could be similar or convergent. We thus tested the hypothesis that maintenance of refined RFs in
121 adulthood depends on the stability of mature receptors and other postsynaptic signaling components. This
122 hypothesis predicts that the detrimental, post-critical period receptive field plasticity observed in DR adult

123 hamsters results from a deprivation-induced failure to maintain these postsynaptic proteins in their mature
124 state. Contrary to this hypothesis, we find, using Western blot assays of synaptosomes, that the quantity,
125 subunit composition, and localization of $GABA_A$ receptors in SC of adult dark reared hamsters with re-
126 expanded RFs resemble those seen in normally reared subjects. Furthermore, levels of the synaptic
127 scaffolding proteins gephyrin and PSD-95 are normal, as are the adult expression levels of cation-chloride
128 co-transporters (KCC2/NKCC) in DR subjects. These findings suggest that, although a change in
129 effectiveness of $GABA_A$ receptors was reported previously using pharmacological agents (Carrasco et al.,
130 2011; Balmer and Pallas, 2015), the loss of RF refinement in adulthood is mediated primarily by
131 reductions in GABA expression in the presynaptic terminals rather than by significant postsynaptic
132 alterations. This result is at odds with the common view that presynaptic changes in the ligand must
133 occur together with corresponding postsynaptic changes in receptor levels (Fisher-Lavie and Ziv, 2013;
134 Sudhof, 2018 ; Sanderson et al., 2020). Taken together, our results rule out several hypotheses about the
135 mechanistic basis of refined RF maintenance throughout adulthood and provide insights into regulation of
136 critical period plasticity that could help to understand the regulation of GABAergic signaling at the
137 synaptic level. Similar research in diurnal animals that have photopic vision as do humans could help to
138 provide insight on treatment and therapeutic modalities in adults facing issues with plasticity in
139 adulthood.
140

141 # Materials and Methods

142 **Subjects**

143 A total of 42 adult Syrian hamsters (Cricetidae: *Mesocricetus auratus*) (aged P90-P100) of both sexes
144 were bred within our animal facility and used as subjects in this study. Syrian hamsters are an altricial
145 rodent species that is ideal for studying the developing visual system due to their robust and well-
146 characterized visual responses, short gestation time, and large litters (Chalupa, 1981; Huck et al., 1988;
147 Pratt and Lisk, 1989; Razak et al., 2003; Carrasco et al., 2005). Sexual maturity in this species occurs

148 between postnatal days (P)56 and P60 (Diamond and Yanagimachi, 1970; Fitzgerald and Zucker, 1976).
149 Breeding females were singly housed. Male breeders were introduced and supervised until intromission
150 was observed, after which they were removed. Weanlings and adult males were housed in single sex
151 social groups of up to 5 adults per cage and adult females were housed with female siblings or
152 individually in standard rodent cages. Running wheels were not provided because they have been shown
153 to alter the timing of visual cortical plasticity (Baroncelli et al., 2010; Tognini et al., 2012; Kalogeraki et
154 al., 2014) but a variety of other enrichment items were available. All subjects were provided with *ad*
155 *libitum* access to rodent chow and water.
156
157 **Treatment groups**
158 Animals used in this study were bred in-house to control sensory experiences throughout development.
159 Dams of DR subjects were transferred into total darkness 1-3 days before parturition. An antechamber
160 and light-impenetrable black curtain separated the dark housing room from the hallway, ensuring that any
161 accidental openings of the hallway doors did not expose the animals to light. Dark reared animals were
162 housed inside light-tight stackable cages with a standard HVAC filtration system consistent with the other
163 animal rooms in the facility. During general animal husbandry purposes, the hamsters were exposed to
164 dim red light at a wavelength not visible to Syrian hamsters (Huhman and Albers, 1994). NR hamsters
165 were maintained in a standard 12/12 light cycle room from before birth into adulthood.
166
167 **Western blotting**
168 Animals were euthanized with Euthasol at >150 mg/kg IP prior to tissue collection. Brains were
169 immediately extracted and frozen in 2-methylbutane on dry ice, then stored at -80°C or immediately
170 dissected for preparation of lysates. In order to analyze differences in protein levels between NR and DR
171 hamsters, we used immunoblotting (Western blots). Western blots can detect protein levels at a 1-3 ng
172 resolution (Coorssen et al., 2002; Ghosh et al., 2014), allowing high resolution quantification of proteins.
173 Note that normal levels of synaptic GABA concentration have been estimated to be between 1.5-3 mM

174 (Coorssen et al., 2002; Tretter et al., 2008; Ghosh et al., 2014). GAPDH or β-actin were used as loading
175 controls to normalize for any differences in the amount of lysate pipetted into each gel lane.
176 Protein extraction was done as described by Shi et al. (1997) with a few modifications. Briefly,
177 individual left and right SC brain areas were excised and homogenized in a lysis buffer (10 mM
178 phosphate buffer, pH 7.0, 5 mM EGTA, 5 mM EDTA, 1 mM DTT) containing Halt protease inhibitor
179 (ThermoFisher Scientific). The lysate was centrifuged at 13,000 rpm at 4°C for 10 min, and the
180 supernatant was saved for the analysis of cytosolic proteins (cytosolic fraction). The resulting pellet was
181 resuspended in 2 mM HEPES buffer, pH 7.2. It was then centrifuged at 70,000 rpm at 4°C for 30 min.
182 The pellet thus obtained was resuspended in 0.5 mM HEPES, pH 7.3, containing 0.32 M sucrose and
183 centrifuged at 2,000 rpm for 8 min. Synaptosomes are present in the supernatant with this method. The
184 success of the synaptosome isolation protocol was confirmed by assessing the presence of Histone H3,
185 which should only be present in the cytosolic fractions and not in the synaptosome fractions **(Figure 2)**.
186 Synaptic proteins were then quantified using the Pierce BCA Protein Assay Kit (ThermoFisher Scientific)
187 mixed with 2X sample buffer and heated for 15 min at 60 °C. Twenty µg of the synaptosome proteins
188 were loaded per well in pre-cast Bio-Rad gels and electrophoresis was carried out at 110 V for 90 min in a
189 Bio-Rad electrophoresis tank. Proteins were then transferred onto nitrocellulose membranes at 70 V for
190 75 min, blocked in BSA for 1 h, and probed with primary antibodies overnight. Primary antibodies used
191 in this study included: rabbit anti-$GABA_A R\alpha 1$ (1:1000, Cat#: AGA-001, Alomone Labs); rabbit anti-
192 $GABA_A R\alpha 2$ (1:1000, Cat#: ab72445, Abcam); rabbit anti- $GABA_A R\alpha 5$ (1:1000, Cat#: ab10098, Abcam);
193 rabbit anti-Gephyrin (1:1000, Cat#: ab32206, Abcam); mouse anti-PSD-95 (1:1000, Cat#: ab2723,
194 Abcam); mouse anti-KCC2 (1:1000, Cat#: 75-013, NeuroMab); rabbit anti-NKCC1 (1:1000, Cat#:
195 ab59791, Abcam); mouse anti-β-actin (1:1000, Cat#: A2228, Sigma-Aldrich) and mouse anti-GAPDH
196 (1:1000, Cat#: 600-GAPDH, PhosphoSolutions). Protein bands were labeled using either appropriate
197 fluorescent secondaries or appropriate HRP-conjugated secondary antibodies, then imaged on an Odyssey
198 CLx fluorescent imaging system (Li-Cor) or developed with enhanced chemiluminescent (ECL) substrate
199 in a Bio-Rad ChemiDoc Imager. All of the proteins studied here were analyzed and quantified as a ratio

200 of the optical density of the protein of interest compared to the density of the loading control (either
201 GAPDH or β-actin). Note that GAPDH is found in the pre-and post-synaptic sites (Frederikse et al., 2016)
202 along with β-actin, and thus makes an effective control protein to measure relative densities.
203
204 **Statistical Analysis**
205 A Student's *t*-test was used to compare parametric data with equal variance between treatment groups and
206 a normally distributed control data set. In the case of non-parametric data (data that were not normally
207 distributed and/or exhibited unequal variance), a Mann-Whitney Rank Sum (U) test was employed.
208 Descriptive statistics for these analyses are provided as mean ± standard error of the mean (SEM) in the
209 text. Whiskers represent the 5th (lower) and 95th (upper) percentage of the data.
210

211 # Results

212 The failure to maintain RF refinement in adult DR animals involves deficits in overall GABA expression
213 and $GABA_A$ receptor function (Carrasco et al., 2011), leading to a loss of lateral inhibition and thus
214 expansion of RFs after P60 (Carrasco et al., 2005; Balmer and Pallas, 2015; Mudd et al., 2019). Using
215 adult hamsters (postnatal day (P)90-P100), we explored several possible ways that dark rearing during a
216 critical period for RF refinement could affect levels of $GABA_A$ receptors and other postsynaptic proteins
217 associated with inhibitory synaptic function in adult SC. We used Western blotting on synaptosomes in
218 normal and dark reared animals to study postsynaptic proteins that might regulate synaptic plasticity.
219

220 **Dark rearing does not affect the subunit composition of $GABA_A$ receptors in adult SC**
221 Deprivation-induced decreases in both $GABA_A$ and NMDA receptor levels in cat visual cortex have been
222 reported previously and were proposed to be involved in functional deficits (Carmignoto and Vicini,
223 1992; Chen et al., 2000; Chen et al., 2001). In DR hamsters, GAD and GABA immunoreactivity declines
224 (Carrasco et al., 2011; Otfinoski & Pallas, in prep.) and $GABA_A$ receptors are less efficient when tested

225 pharmacologically (Carrasco et al., 2011), thus we expected to see changes in levels of postsynaptic
226 $GABA_A$ proteins. However, in our previous study using Western blots on synaptosomes, no significant
227 changes in the level of the $GABA_A$ receptor alpha1 subunit were observed (Mudd et al., 2019). These
228 results raised the question of whether subunit composition of $GABA_A$Rs might be altered by dark rearing
229 in a way that reduced their effectiveness without affecting alpha1 levels.

230 We reasoned that the developmental alpha2 to alpha1 switch, if reversed in adulthood, could underlie
231 the reduction in $GABA_A$ receptor function that was previously observed in studies of RF enlargement in
232 adult SC (Carrasco et al., 2011). We explored this possibility by examining the expression of each subunit
233 in synaptosomes of SC in normally reared and visually deprived adults. Hamsters in the experimental
234 group were dark reared from before birth. We used Western blotting for a high resolution, quantitative
235 assay of synaptic membrane-bound alpha1 and alpha2 $GABA_A$ receptor expression in the synaptosome
236 fractions obtained from adult SC. We found that there were no significant differences in either the overall
237 levels of alpha2 protein, observed as a ratio of alpha2 to GAPDH (NR: 1.21 ± 0.052, n=8; DR: 1.33 ±
238 0.185, n=8; U=26, n=8, p=0.574; Mann-Whitney Rank Sum Test) **(Figure 3A)**, or in the ratio of
239 alpha1/alpha2 expression, observed as a ratio of the normalized alpha 1 density (alpha 1/GAPDH ratio) to
240 normalized alpha 2 density (alpha 2/GAPDH ratio) in the SC of adult DR (1.651 ± 0.277, n=5) compared
241 to adult NR hamsters (1.19± 0.084, n=4) (T(7)= -1.436, n=4, p=0.194 Student's t-test) **(Figure 3B)**. This
242 was a surprising result considering our previous finding that dark rearing reduces the response to
243 pharmacological application of GABA agonists and antagonists (Carrasco et al., 2011). These findings
244 argue against a reversal of the normal developmental transition from alpha2 to alpha1-dominant
245 expression as a cause of the deprivation-induced RF enlargement in adult SC, and they support the
246 interpretation that visual experience is not needed to maintain mature $GABA_A$ receptor alpha1/alpha2
247 subunit composition.

248 $GABA_A$ receptors can also be expressed extrasynaptically, where they can be activated by GABA
249 derived from synaptic spillover or non-neuronal sources. This low concentration GABA source generates
250 "tonic" inhibition (Farrant and Nusser, 2005). Alpha5 subunit-containing receptors are primarily

251 expressed extrasynaptically and have been implicated in regulating the induction of synaptic plasticity for
252 LTP in hippocampus (Saab et al., 2010; Zurek et al., 2012; Zurek et al., 2014; Jacob, 2019). However,
253 alpha5 $GABA_A$Rs can relocate to the synapse and colocalize with gephyrin (Brady and Jacob, 2015). To
254 investigate the possible role of synaptic alpha5 levels in adult RF maintenance we quantified and
255 compared the alpha5/GAPDH ratios between NR (0.544 ± 0.0520, n=8) and DR (0.471 ± 0.0935, n=8)
256 adult hamsters **(Figure 4A)** using Western blotting. We found no significant differences in alpha5 protein
257 levels between groups (U (20) = 0.308, p = 0.878, Mann-Whitney Rank Sum test). We compared the ratio
258 of alpha1/alpha5 between adult NR (1.027 ± 0.0815, n=10) and DR (0.995 ± 0.0926, n =9) hamsters and
259 found no differences between these groups (U (18) =35, p=0.438, Mann-Whitney Rank Sum test) **(Figure**
260 **4B)**. Because we were only studying proteins from synaptosome preparations (i.e., $GABA_A$ receptor
261 subunits localized in the synapses), these results suggest that the localization of alpha1 and alpha5
262 subunit-containing $GABA_A$ receptors in SC is not being altered by early visual experience.
263

264 **Dark rearing does not affect the normal location of $GABA_A$ receptors in adult SC**

265 The regulation of $GABA_A$ receptors at the synapse is pivotal for maintaining correct levels of inhibitory
266 synaptic transmission (Jacob et al., 2008). Impaired trafficking of $GABA_A$ receptors into and out of
267 synaptic membranes could affect their synaptic localization in SC and thus their overall response to
268 presynaptically released GABA. $GABA_A$ receptor trafficking is partially regulated by endocytosis – the
269 controlled removal of receptors from the postsynaptic membrane into the cytoplasm (see Lévi and Triller,
270 2006, for review). Endocytosed receptors are subsequently reinserted into the postsynaptic membrane or
271 undergo lysosomal degradation (Kittler et al., 2000). We reasoned that if internalization was dysregulated,
272 either by decreased receptor reinsertion or increased receptor degradation, it could negatively impact the
273 efficacy of $GABA_A$ receptors at the synapse. We examined this possibility by comparing the ratio of
274 postsynaptic membrane-bound to cytosolic alpha1 subunit- containing receptors between our treatment
275 groups. No differences were observed in the postsynaptic membrane/cytosol ratio of alpha1 expressing
276 receptors between DR (0.768 ± 0.044, n=7) and NR (0.778 ± 0.1, n=6) adult hamsters (U(11)= 20.00,

277 p=0.945, Mann-Whitney Rank Sum test) **(Figure 5A)**. These results indicate that the overall trafficking of
278 alpha1 subunit-expressing synaptic $GABA_A$ receptors is not affected by dark rearing.
279 We also examined the possibility that extrasynaptic alpha5 receptor internalization may be
280 dysregulated and thus responsible for changes in tonic $GABA_A$ inhibition (Davenport et al., 2021). There
281 were no significant differences in cytosolic membrane localization of alpha5 subunits between adult DR
282 (1.320 ± 0.198, n=8) and NR groups, however (1.753 ± 0.449, n=6) (U(12)=19.00, p=0.573, Mann-
283 Whitney Rank Sum test) **(Figure 5B)**. These results indicate that the internalization of extrasynaptic
284 alpha5 subunit-expressing $GABA_A$ receptors is not responsible for the decreased efficacy of $GABA_A$
285 receptors observed in RFs that fail to maintain refinement in adulthood after dark rearing.
286
287 **Inhibitory and excitatory scaffolding proteins in SC are not affected by dark rearing**
288 One factor influencing the accumulation and retention of $GABA_A$ receptors at postsynaptic sites is the
289 membrane scaffolding protein gephyrin (Kneussel et al., 1999; Sun et al., 2004; Jacob et al., 2005; Tretter
290 et al., 2008). Decreased expression of gephyrin results in less clustering (Essrich et al., 1998) and more
291 mobility of $GABA_A$ receptors at the synapse (Jacob et al., 2005). We surmised that decreased gephyrin
292 expression could be responsible for the weaker $GABA_A$ receptor signaling observed in neurons with RFs
293 that failed to maintain refinement in adulthood. We quantified and compared postsynaptic membrane-
294 bound gephyrin expression between DR and NR adults using Western blotting. Gephyrin levels in DR
295 adults (0.786 ± 0.124, n=17) were similar to those in NR adults (0.736 ± 0.158, n=16) (U(111)=-0.247,
296 p=0.377, Mann-Whitney Rank Sum test) **(Figure 6A)**. This indicates that maintenance of adult gephyrin
297 expression levels is not affected by dark rearing and suggests that if $GABA_A$ receptor accumulation and
298 trafficking is being affected, then it is occurring independently of gephyrin levels.
299 PSD-95 is the primary glutamate (AMPA and NMDA) receptor scaffolding protein in CNS neurons
300 (Chen et al., 2015), and it functions like gephyrin does for $GABA_A$ receptors. Although it does not
301 directly impact $GABA_A$ receptor function, PSD-95 has an influence on visual circuit plasticity. For
302 example, mice lacking PSD-95 have lifelong ocular dominance plasticity in primary visual cortex that

303 results from an increase in the overall proportion of silent synapses, despite having normal inhibitory tone
304 (Funahashi et al., 2013; Huang et al., 2015). Thus, we examined whether the dark rearing-induced re-
305 enlargement of RFs could be mediated by a reduction in adult PSD-95 expression. We found that PSD-95
306 protein levels were not significantly different in DR (0.613± 0.96, n=10) compared to NR adult hamsters
307 (0.486 ± 0.868, n=9) (U(31)=-0.978, p=0.270, Mann-Whitney Rank Sum test) **(Figure 6B)**. These results
308 suggest that differences in PSD-95 levels do not underlie the re-enlargement of RFs in SC following dark
309 rearing from birth.
310
311 **Cation-chloride co-transporters undergo their normal developmental switch in adult dark reared**
312 **subjects**
313 Levels of inhibitory GABAergic signaling in neurons are dependent on the intracellular chloride (Cl^-)
314 concentration. The K^+ Cl^- co-transporter (KCC2) is responsible for regulating intracellular Cl^- in mature
315 adult neurons with an outward K^+ current (Rivera et al., 1999) and also regulates the formation, function,
316 and plasticity of glutamatergic synapses (Li et al., 2007; Gauvain et al., 2011; Chevy et al., 2015). At the
317 beginning of postnatal life, $GABA_A$ receptor effects are excitatory because the Na^+-K^+-$2Cl^-$ co-
318 transporter 1 (NKCC1) that mediates Cl^- uptake is dominant (Cherubini et al., 1991; Lee et al., 2005;
319 Cancedda et al., 2007). By the end of the second postnatal week in rats and mice NKCC1 is replaced by
320 KCC2 as the dominant cation-chloride co-transporter in the brain, shifting the resting membrane potential
321 and thus causing $GABA_A$ receptors to produce inhibitory PSPs (Rivera et al., 1999; Pfeffer et al., 2009;
322 Moore et al., 2019). In V1, the developmental switch from NKCC1 dominance to KCC2 dominance
323 occurs at the same time as a period of BDNF/TrkB mediated synaptic imbalance (Zhang et al., 2018). We
324 surmised that a shift in the ratio of KCC2:NKCC1 could underlie the reinstatement of RF size plasticity in
325 dark reared adults, leading to re-enlargement of RFs in SC. Examination of the expression of KCC2 and
326 NKCC1 in adult SC neurons revealed no significant differences between our treatment groups, however.
327 KCC2 levels were not significantly different between NR (0.979 ± 0.115, n=8) and DR groups of adult
328 hamsters (0.963 ± 0.154, n=8) (T(14)=0.082, p=0.936, t-test) **(Figure 7A)**. The same was true of NKCC1

329 levels (NR 1.050 ± 0.0419, n=8; DR 1.081 ± 0.0814, n=8) (T(14)=-0.339, p=0.740, t-test) **(Figure 7B)**,
330 and of the ratio of the two cation-chloride co-transporters within groups (T(8)=1.096, p=0.305, t-test)
331 **(Figure 7C)**.
332

333 **Discussion**

334 The goal of this study was to examine potential postsynaptic mechanisms through which light exposure
335 during an early critical period ensures the long-term stability of visual receptive fields in the hamster
336 superior colliculus. Our previous results established a correlation between the maintenance of RF
337 refinement and levels of GABA immunoreactivity in SC (Carrasco et al., 2011; Mudd et al., 2019) and
338 V1 (Balmer and Pallas, 2015), but potential postsynaptic changes in $GABA_A$ receptor and related protein
339 levels had not been examined. We have reported here that at the high-resolution level of Western blot
340 protein quantification, visual deprivation-induced failure to maintain refined RFs in SC does not appear to
341 involve changes in $GABA_AR$ subunit composition, inhibitory or excitatory scaffolding protein expression,
342 or cation-Chloride co-transporter ratios. These results exclude several possible mechanisms that could
343 explain the reduced activation of $GABA_ARs$ with GABA agonists reported in DR adult SC (Carrasco et
344 al., 2011), and support activity-dependent regulation of GABA expression as the primary mechanism
345 underlying TrkB-mediated maintenance of RF refinement (Mudd et al., 2019). The finding that a change
346 in GABA levels could affect RF refinement in adulthood has important implications for the treatment of
347 memory impairments or brain injury.

348 This study supports our previous research that provided substantial evidence of a novel, maladaptive
349 adult plasticity in which visually deprived hamsters refine SC RFs normally but fail to maintain them in
350 adulthood. Our research differs from these previous studies in suggesting that dark reared hamsters lose
351 visual refinement in adulthood and not, as in the case of monkeys, ferrets, and cats, during development
352 (Mower and Christen, 1985; Mower et al., 1986; Mower, 1991; Carmignoto and Vicini, 1992; Fagiolini et
353 al., 1994; Chen et al., 2000; Chen et al., 2001; Lee and Nedivi, 2002; Erisir and Harris, 2003). Some of
354 these previous studies looked only at early and/or adult ages in the animals, thereby missing the RF

355 refinement that happens in between the two ages. Because diminished GABA release, contrary to what
356 we expected, did not elicit measurable changes in the levels of postsynaptic $GABA_A$ receptors, scaffold
357 proteins, or chloride co-transporters, this study provides a valuable demonstration that changes in
358 neurotransmitter availability do not necessarily result in coordinated changes in postsynaptic receptors.
359 Maturation of GABAergic signaling in visual cortex, particularly of the fast-spiking, parvalbumin-
360 containing basket cells, is thought to open and then close the critical period for plasticity (Fagiolini et al.,
361 2004; Sale et al., 2010; Toyoizumi et al., 2013; Capogna et al., 2021). Combined pre- and postsynaptic
362 alteration of synaptic strength has been seen in other sensory deprivation paradigms, including in dark
363 reared and monocularly deprived visual cortex, although with an earlier time course (Carmignoto and
364 Vicini, 1992; Chen et al., 2000; Chen et al., 2001). However, the retinorecipient layers of the superior
365 colliculus have no basket cells and contain very few GABAergic parvalbumin neurons, and the plasticity
366 described here occurs after the critical period has closed, suggesting that SC may accomplish plasticity
367 through a different mechanism than visual cortex. On the other hand, previous studies found that, as in
368 visual cortex (Hanover et al., 1999; Huang et al., 1999; Viegi et al., 2002), deprivation-induced receptive
369 field plasticity in adult SC is mediated by the BDNF receptor TrkB (Mudd et al., 2019). Furthermore,
370 reduced GABA and $GABA_A$ receptor efficacy in response to iontophoretically-applied $GABA_AR$ agonists
371 and antagonists is observed in both SC and V1 of dark reared hamsters (Carrasco et al., 2011; Balmer and
372 Pallas, 2015), arguing for mechanistic elements in common.
373
374 ***Early visual experience is not necessary for maturation or maintenance of $GABA_A$ receptor subunit***
375 ***composition at the synapse***
376 $GABA_A$ receptors contain fast acting chloride (Cl^-) channels (Pfeiffer et al., 1982; Sigel and
377 Steinmann, 2012). The subunit composition of $GABA_A$ receptors changes during development from an
378 alpha 2 to alpha 1 dominant condition (Laurie et al., 1992; Fritschy et al., 1994; Chen et al., 2001) and
379 also changes in some disease states (Levitt, 2005; Tyson and Anderson, 2014; Deidda et al., 2015;
380 Kimoto et al., 2015; Schmidt and Mirnics, 2015; Tang et al., 2021) in a way that affects receptor

381 functional properties (Farrant and Nusser, 2005) and localization (Jacob et al., 2005). We studied synaptic
382 levels of the $GABA_A$ receptor alpha1 and alpha2 subunits to quantify their expression levels under normal
383 and DR conditions. The normalized expression levels of $GABA_AR$ alpha1 relative to $GABA_AR$ alpha2
384 levels were not altered in DR hamsters when compared to those of NR hamsters, arguing that the altered
385 inhibitory synaptic efficacy that we previously observed was not caused by an immature $GABA_AR$
386 subunit composition at the synapse.

387 An increase or decrease in the level of any receptor subunit is best understood in context, because
388 different conclusions would be drawn if subunits changed independently or in concert. Thus, we also
389 analyzed the alpha1/alpha2 ratios in individual animals. We did not find any change in alpha1/alpha2
390 ratios in NR compared to DR adult hamsters. These results suggest that early visual experience is not
391 necessary for maturation or maintenance of mature synaptic $GABA_AR$ subunit composition in adulthood.
392 Thus, the failure to maintain refined RFs in adult DR hamsters cannot be explained by a return to a
393 juvenile type of $GABA_AR$ subunit composition.

394

395 ***Level and localization of the extrasynaptic $GABA_AR$ subunit alpha 5 does not change with dark***
396 ***rearing***

397 $GABA_AR$ subunit alpha 5 is predominantly an extrasynaptic membrane receptor subunit that regulates
398 tonic inhibition. It is important in neuronal circuit development, learning, and memory (Brady and Jacob,
399 2015), has been implicated in regulating the induction of synaptic plasticity in hippocampus (Saab et al.,
400 2010; Zurek et al., 2012; Zurek et al., 2014), and can relocate to the synaptic region in learning and
401 memory deficits (Brady and Jacob, 2015). Because the excitation/inhibition (E/I) balance could be
402 affected if alpha5 subunit levels changed or if they moved into the synapse, we compared its expression
403 between NR and DR cases. We did not see any significant changes in the overall levels of $GABA_AR$
404 alpha5 subunits, or in the alpha1/alpha5 ratio, suggesting that dark rearing-induced RF enlargement is not
405 caused by changes in the $GABA_AR$ subunit alpha 5 levels or localization in the synapse.

406

407 ***Dark rearing does not affect the rate of internalization of $GABA_AR$ subunits at the synapse***

408 Because clathrin-dependent endocytosis is likely important for regulating inhibitory signaling and
409 synaptic plasticity (Kittler et al., 2000), we explored the internalization of $GABA_A$ receptor alpha1 and
410 alpha5 subunits by comparing their synaptic vs. extrasynaptic location in normally reared and dark reared
411 subjects. We did not observe a significant change in location of either subunit type as assayed by the ratio
412 of synaptosome-bound to cytosolic fractions. This implies that a lack of visual experience does not affect
413 the trafficking of the $GABA_AR$ subunits between the synaptic membrane and the cytosol or the
414 phosphorylation events that maintain the balance between internalization and postsynaptic membrane
415 insertion of the receptor subunits.

416

417 ***Early visual experience is not necessary to maintain scaffolding protein levels at the synapse***

418 Another finding of this work is that the expression levels of the postsynaptic scaffold proteins PSD-
419 95 and gephyrin were not altered in adulthood following lifelong lack of light exposure, suggesting that
420 any changes in inhibitory function are probably not caused by a significant change in scaffolding protein
421 expression. At any rate, the clustering of $GABA_ARs$ at inhibitory synapses in SC may happen in a
422 gephyrin-independent manner (Kneussel et al., 2001), or total gephyrin expression may not be as
423 important as the formation of gephyrin nanodomains within inhibitory synapses (Pennacchietti et al.,
424 2017). Future studies with additional techniques would be required to determine if changes in receptor
425 clustering may be occurring and what role gephyrin or PSD-95 may have in mediating any such effects.

426

427 ***Early light exposure is not necessary for maturation of the cation-chloride co-transporters***

428 We investigated the status of the chloride transporters KCC2 and NKCC1 due to their role in maintaining
429 chloride balance inside of the neurons and thus in setting the reversal potential. The cation-chloride co-
430 transporters could have reverted to their early developmental state, leading to a lower threshold for
431 excitation in dark reared animals, possibly explaining the RF expansion we observed. However, we did
432 not observe any changes in the cation-chloride co-transporters in dark-reared compared to normally

433 reared adult hamsters, suggesting that the RF enlargement was not caused by alterations in the cation-
434 chloride co-transporters.
435
436 ***A GABA-BDNF feedback loop maintains inhibitory networks, thereby maintaining RF refinement in***
437 ***adulthood***
438 GABA-$GABA_A$R interaction is known to regulate various downstream signaling pathways, and a
439 major regulator of GABA itself is BDNF-TrkB signaling triggered by NMDA receptor activity (Marini et
440 al., 1998). Our data are consistent with previous studies suggesting a positive feedback loop between the
441 BDNF-TrkB pathway and GABA expression, in which GABA facilitates BDNF expression, and BDNF
442 facilitates the production of GABA by GAD (Sánchez-Huertas and Rico, 2010) and its synaptic release
443 (Huang et al., 1999; Morales et al., 2002; Gianfranceschi et al., 2003; Jovanovic et al., 2004; Kuczewski
444 et al., 2008; Porcher et al., 2011; Hanno-Iijima et al., 2015), maintaining RF size and visual acuity
445 through GABAergic lateral inhibition (Mudd et al., 2019). Signaling via the MAPK cascade and the
446 transcription factor cAMP-responsive element-binding protein (CREB) appears to play a substantial role
447 in this process (Obrietan et al., 2002; Sánchez-Huertas and Rico, 2010). BDNF-TrkB interaction leads to
448 dimerization and auto-phosphorylation of TrkB, thereby triggering MAPK, PLC gamma, and PI3K
449 pathways (Yoshii and Constantine-Paton, 2007). These pathways in turn lead to the activation of
450 downstream effectors and mediators to initiate a CREB-dependent transcription process that can lead to
451 an increase in $GABA_A$R levels as well as more BDNF production (Huang and Reichardt, 2003; Yoshii
452 and Constantine-Paton, 2007; Porcher et al., 2011; Esvald et al., 2020). In addition, an increase in the
453 transmembrane localization of $GABA_A$Rs is mediated by BDNF-dependent inhibition of receptor
454 internalization in addition to ongoing reinsertion of the receptor into the postsynaptic membrane (Porcher
455 et al., 2011). This positive feedback regulation is critical in developing neurons and hence constituted a
456 major motivation for the work reported here. In this study however, neither the $GABA_A$R subunit
457 composition at the synapse nor subunit composition in the extrasynaptic regions was affected by dark
458 rearing. Chloride transport proteins (KCC2, NKCC2) also remained at normal levels. One possibility is

459 that GABA expression levels alone are the key factor in RF re-enlargement in hamster SC (Carrasco et
460 al., 2011; Mudd et al., 2019). If so, it would suggest that this type of delayed plasticity resulting from a
461 lack of early visual experience occurs through a different mechanism than described in other types of
462 plasticity resulting from dark rearing (Mower et al., 1985; Chen et al., 2000; Chen et al., 2001).

463 It is possible that changes in $GABA_AR$ signaling occurred that are not reflected here in the expression
464 levels of postsynaptic receptor composition, scaffolding molecules, or ion transporters, or that we missed
465 some transient changes in $GABA_AR$ signaling-associated proteins that cause $GABA_AR$ functional
466 changes. In the future, it would be interesting to study protein localization and interactions with
467 immunohistochemistry, or to study the properties of synaptic and extrasynaptic responses in the SC with
468 patch clamp experiments, and the subcellular dynamics of associated proteins involved. This might
469 improve the understanding of the molecular processes active in this deprivation-induced, maladaptive
470 plasticity in the SC.

471 Suggested alternative explanations include that the changes leading to RF expansion and thus the
472 visual acuity deficits take place earlier than the time point that we studied and return to normal by P90.
473 The width of the synaptic cleft decreases during development (Li and Cline, 2010), thus, another
474 interesting possibility is that dark rearing from birth gradually increases the width of the synaptic cleft in
475 adulthood while keeping the postsynaptic signaling components in place. Alternatively, the synapses may
476 be present but silent due to the presynaptic loss of GABA (Carrasco et al., 2011). It is also possible that
477 other scaffolding proteins and their partners are involved. Potential candidates include gephyrin binding
478 partners such as $GABA_AR$ beta2 and beta3 subunits (Kowalczyk et al., 2013), the scaffold protein radixin
479 that binds $GABA_AR$ alpha5 subunit to the actin cytoskeleton (Loebrich et al., 2006) and regulates
480 synaptic $GABA_AR$ density (Hausrat et al., 2015), or gephyrin post-translational modification events that
481 influence inhibitory synaptic plasticity by affecting postsynaptic scaffolding (Zacchi et al., 2014). More
482 recently, distinct spectrin isoforms have been shown to affect synaptic inhibition by selectively targeting
483 specific $GABA_AR$ subunits, including α1 and α2, to particular regions of the neuron (Smalley et al.,
484 2023).

485 In summary, our results argue that visual experience is not necessary to maintain mature levels and
486 composition of several postsynaptic proteins that are essential for retino-SC synaptic communication.
487 Unlike many previous dark rearing studies in which both GABA and $GABA_A$ receptors were found to be
488 downregulated (Carmignoto and Vicini, 1992; Chen et al., 2000; Chen et al., 2001; Kilman et al., 2002;
489 Nahmani and Turrigiano, 2014), we report here that in the SC, $GABA_AR$ levels, subunit composition, and
490 localization in adulthood are unaffected by dark rearing. The scaffold proteins gephyrin and PSD-95, and
491 the chloride transporters KCC2 and NKCC2 are also not affected by dark rearing. This novel,
492 experience-dependent form of adult plasticity may involve an as yet unidentified postsynaptic
493 mechanism, or only a reduction in GABA release (Carrasco et al., 2005; Carrasco and Pallas, 2006;
494 Carrasco et al., 2011), thereby challenging the common view that presynaptic changes in ligand
495 availability are always associated with matching postsynaptic changes in their receptors. Either possibility
496 is encouraging with respect to understanding this form of adult plasticity and might help adults with
497 memory impairments, traumatic brain injury, or inhibition-associated neurological disorders.
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791

792

793 **Figure legends:**

794

795 **Figure 1: Differences in the visual refinement of hamster visual pathways depending on exposure to**
796 **light during the critical period of heightened neural plasticity.** Animals exposed to light during postnatal
797 development gradually improve RF refinement in SC and V1 and maintain it throughout life (as indicated
798 by the blue line). Animals that do not experience postnatal light also show RF refinement by P60, but the
799 refinement progressively declines in adulthood (as indicated by the orange line).

800

801 **Figure 2. Histone H3 expression in the synaptosomal and cytosolic lysates.** Western blot showing
802 histone H3 (15 kDa) bands in the synaptosomal and cytosolic fractions of SC. GAPDH was used as a
803 loading control. The presence of histone H3 in the cytosolic fraction and its absence in the synaptosomal
804 fraction shows an effective synaptosomal separation occurred in these experiments.

805

806 **Figure 3. $GABA_A$α2 receptor subunit levels in SC are not affected by early dark rearing. (A) Image:**
807 Individual Western blots of normally reared (NR) and dark-reared (DR) treatment groups generated using
808 20 µg of SC protein per lane. GAPDH was used as a loading control. **Plot:** Boxplot showing the
809 normalized $GABA_A$Rα2 expression level in normally reared vs. dark reared hamsters. **(B) Image:**
810 Individual Western blots of SC tissue from NR and DR animals comparing $GABA_A$Rα1 and $GABA_A$Rα2
811 expression with corresponding GAPDH expression. **Plot:** Boxplot showing the ratio of normalized values
812 of $GABA_A$Rα1/GAPDH to the normalized values of $GABA_A$Rα2/GAPDH. Boxes in each individual
813 boxplot show the median and 25th and 75th percentiles of the data (whiskers show 5% and 95% levels).
814 Individual data points obtained from each animal within a group are shown as dots. For Western blots in
815 this and the following figures, lanes presented together are from the same gel, and each measured protein
816 was normalized against GAPDH (unless stated otherwise) as a loading control. Taken together, these
817 results reveal that the levels and ratio of synaptic $GABA_A$Rα2 receptor subunits and their ratio with
818 $GABA_A$Rα1subunits are similar in normal and dark reared adult SC.

819

820 **Figure 4. $GABA_A\alpha5$ receptor subunit levels in SC are not affected by early dark rearing. A and B**
821 **Images:** Representative Western blots of NR and DR treatment groups as in Figure 3. All lanes presented
822 together are from the same gel(s), and each gel was run with GAPDH as a loading control. **(A) Plot:**
823 Boxplot showing normalized $GABA_AR\alpha5$ expression levels compared between normal and dark reared
824 hamsters. **(B) Plot:** Boxplot showing the ratio of normalized (against GAPDH levels) $GABA_AR\alpha1$:
825 $GABA_AR\alpha5$ expression ratios. Boxes in each individual boxplot show the median and 25th and 75th
826 percentiles of the data, with whiskers at 5 and 95%. Individual data points in each group are shown as
827 dots. These results show that the levels of synaptic $GABA_A\alpha5$ receptor subunits and their ratio with
828 $GABA_AR\alpha1$ subunits are similar in normal and dark reared adult SC.

829

830 **Figure 5. Internalization of $GABA_A$ receptors in SC is not affected by early dark rearing. (A)** Adult
831 levels of the cytosolic vs. the synaptic membrane-attached ratio of the synaptically-targeted $GABA_AR\alpha1$
832 and **(B)** the synaptically-targeted $GABA_AR\alpha5$ subunits were not affected by early light deprivation.
833 **Images:** Representative Western blots represent bands of cytoplasmic and membrane bound receptor
834 subunit proteins, each from the same animal, measured as a ratio against GAPDH and compared between
835 NR and DR groups. **Plots:** Boxplot showing the ratio of normalized (against GAPDH) values of cytosol:
836 membrane ratios of each subunit. Boxes in each individual boxplot show the median and 25th and 75th
837 percentile of the data, with whiskers indicating 5 and 95%. Individual data points obtained in each group
838 are shown as dots. These results show that the internalization of synaptic $GABA_AR\alpha1$ and $GABA_AR\alpha5$
839 subunits is similar in normal and dark reared adult SC.

840

841 **Figure 6. Gephyrin and PSD-95 expression in SC are not affected by dark rearing. Images: (A)**
842 Gephyrin and **(B)** PSD-95 expression was similar between adult NR and DR groups (upper panels).
843 **Plots:** Boxplot showing the ratio of normalized values of gephyrin vs. GAPDH (A) or β-actin (B) . Boxes
844 in each individual boxplot show the median and 25th and 75th percentiles of the data, with whiskers at 5

845 and 95%. Individual data points obtained in each group are shown as dots. These results show that the
846 levels and ratio of the scaffold proteins are similar in normal and dark reared adult SC.
847
848 **Figure 7. Cation-chloride co-transporter expression in SC is not affected by early dark rearing.**
849 **Images:** Example Western blots of NR and DR samples labeled for cation-chloride co-transporters **(A)**
850 KCC2 (140 kDa) and **(B)** NKCC1 (150 kDa) compared to the GAPDH loading control and **(C)** a
851 comparison of the within subject ratio of KCC2:NKCC1 in NR and DR adult hamsters. **Plots:** Boxplots
852 showing the levels of KCC2 and NKCC1 proteins, normalized against GAPDH **(A and B, respectively)**
853 and comparison of normalized values of KCC2/GAPDH to that of NKCC1/GAPDH **(C)**. Boxes in each
854 boxplot show the median, 25th and 75th percentiles of the data. Whiskers are at 5 and 95% percentiles.
855 Individual data points obtained in each group are shown as dots. These results show that the number and
856 ratio of cation chloride co-transporters are similar in normal and dark reared adult SC.