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

Abbreviated title: Postsynaptic proteins unaffected by dark rearing

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Abstract

Dark rearing in kittens and monkeys delays visual pathway development and prolongs the critical period. In contrast, receptive fields (RFs) in superior colliculus (SC) and visual cortex (V1) of Syrian hamsters refine normally with spontaneous activity alone, requiring only brief juvenile visual experience to maintain refined RFs in adulthood. Continued visual deprivation into adulthood reduces GAD and GABA levels due to reduced BDNF-TrkB signaling, resulting in RF re-enlargement. To explore possible postsynaptic changes caused by dark rearing, we examined expression of $GABA_A$ receptors and their anchoring protein gephyrin and cation-chloride co-transporters KCC2 and NKCC1 in dark reared hamsters. We found that dark rearing from birth until puberty had no effect on levels of postsynaptic $GABA_A$ receptors or their subunit composition, anchoring proteins, or chloride transporters. Thus, visual deprivation from birth to puberty reveals a new form of maladaptive, inhibitory plasticity in which, rather than extending the critical period, 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.

Contribution to the field statement

Raising animals in darkness from birth prevents development of the visual system in some mammals. In Syrian hamsters, however, the visual system develops normally in the dark, but continued visual deprivation into adulthood leads to a gradual re-expansion of receptive field size. We showed previously that a loss of presynaptic inhibition contributes to the reversal of visual refinement in adulthood. However, the postsynaptic mechanisms that contribute to the reversal of visual refinement are not yet known. Here we show that despite the reduction in GABAergic lateral inhibition there are no detectable changes in postsynaptic $GABA_A$ receptor levels or in several other postsynaptic proteins. These results

show that changes in presynaptic ligand levels do not always correspond with changes in their postsynaptic receptors. They suggest that in some species dark rearing extended into adulthood can re-establish plasticity, potentially producing negative effects on visual acuity.

Introduction

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

In some mammals, dark rearing is reported to delay or prevent refinement, prolonging critical period plasticity (Cynader and Mitchell, 1980; Mower et al., 1985; Mower, 1991; Lee and Nedivi, 2002; Nakadate et al., 2012). In apparent contradiction to this common view, we have reported that spontaneous activity is sufficient for refinement of receptive fields in both visual cortex (V1) and superior colliculus (SC) of dark reared (DR) Syrian hamsters, and early light exposure for 3-7 days is necessary only to maintain the refinement in adulthood (Carrasco et al., 2005; Carrasco and Pallas, 2006; Balmer and Pallas, 2015b; Mudd et al., 2019). Syrian hamster pups spend the first 3-4 weeks after birth underground in the wild (Adler, 1948; Nowosielski-Slepowron and Park, 1987), so it would not be beneficial to have the development of visual function depend on light exposure. If early visual experience is unavailable, GABAergic lateral inhibition in SC and V1 declines and RFs expand, but not until about two months of age (puberty) (Carrasco et al., 2005; Balmer and Pallas, 2015b; Mudd et al., 2019).

101 Pharmacological activation of TrkB receptors can mimic the effects of early light exposure in DR
102 hamsters, resulting in long-term maintenance of refined receptive fields and visual acuity (Mudd et al.,
103 2019). Thus, hamsters, unlike cats and monkeys, need visual experience to maintain refined receptive
104 fields in adulthood, but not to refine them during development **(Figure 1)**.

105 $GABA_A$ receptors are pentameric, ionotrophic receptors consisting of five subunits grouped around a
106 central chloride ion pore. The functional characteristics of the receptor largely depend upon the subunit
107 composition (Sigel et al., 1990) and organization (Minier and Sigel, 2004). Of the many subunit
108 arrangements, alpha1 and alpha2 subunits have been linked to synaptic localization of $GABA_A$ receptors.
109 However, these two subunit types have different kinetics and are expressed at different points in
110 development. At birth, receptors containing the alpha2 subunit are widely expressed throughout the brain,
111 whereas alpha1 expression is low in major areas of the brain like the neocortex, the hippocampus, and the
112 cerebellum (Laurie et al., 1992; Fritschy et al., 1994; Dunning et al., 1999; Chen et al., 2001). During the
113 first several postnatal weeks, alpha1 expression increases, coinciding with a reduction in alpha2
114 expression (Fritschy et al., 1994). This alpha2 to alpha1 switch in subunit expression underlies a
115 developmental decrease in inhibitory postsynaptic current (ipsc) decay time and an increase in ipsc
116 amplitude (Fritschy et al., 1994; Okada et al., 2000; Yu et al., 2006).

117 It was demonstrated previously that the expansion of RFs in SC of adult, DR hamsters is associated
118 with a loss of GABA immunoreactivity. Iontophoretic application of $GABA_A$ agonists *in vivo* restored
119 RFs to a normal adult size (Carrasco et al., 2011). In addition to a loss of GABA-immunoreactivity,
120 $GABA_A$ agonists and antagonists were less effective in SC and V1 neurons of DR hamsters than in
121 normally reared (NR) hamsters (Carrasco et al., 2011).

122 The incomplete development of RF properties in V1 of visually deprived cats has been associated
123 with a failure in developmental maturation of NMDA and $GABA_A$ receptors (Carmignoto and Vicini,
124 1992; Chen et al., 2000; Chen et al., 2001; Erisir and Harris, 2003). Although a failure to maintain
125 refined RFs is a different phenomenon than a failure to refine them during a critical period, the
126 mechanism(s) could be similar or convergent. Here, we provide molecular evidence complementing our

127 previous work (Carrasco et al., 2005; Carrasco and Pallas, 2006; Balmer and Pallas, 2015b) that suggests
128 the presence of divergent synaptic development mechanisms between hamsters and some other mammals
129 (Regal et al., 1976; Timney et al., 1978; Leventhal and Hirsch, 1980; Mower et al., 1981; Morales et al.,
130 2002; Viegi et al., 2002) in regulating RF development and maintenance under dark rearing conditions.
131 We thus tested the hypothesis that maintenance of refined RFs in adulthood depends on the stability of
132 mature receptors and other postsynaptic signaling components. This hypothesis predicts that the
133 detrimental, post-critical period receptive field plasticity observed in DR adults results from a
134 deprivation-induced failure to maintain these postsynaptic proteins in their mature state. Contrary to this
135 prediction, we find using Western blot assays of synaptosomes that the quantity, subunit composition, and
136 localization of $GABA_A$ receptors in SC of adult dark reared hamsters with re-expanded RFs resemble
137 those seen in normally reared subjects. Furthermore, levels of the synaptic scaffolding proteins gephyrin
138 and PSD-95 are normal, as are the adult expression levels of cation-chloride co-transporters
139 (KCC2/NKCC) in DR subjects. These findings suggest that, although a change in effectiveness of
140 $GABA_A$ receptors was reported previously using pharmacological agents (Carrasco et al., 2011; Balmer
141 and Pallas, 2015a), the loss of RF refinement in adulthood is mediated primarily by reductions in GABA
142 expression in the presynaptic terminals rather than by substantial postsynaptic alterations. This result is at
143 odds with the common view that presynaptic changes in the ligand must occur together with
144 corresponding postsynaptic changes in receptor levels (Fisher-Lavie and Ziv, 2013; Sudhof, 2018 ;
145 Sanderson et al., 2020). Taken together, our results rule out several hypotheses about the mechanistic
146 basis of maintenance of refined RFs throughout adulthood, and provide insights into regulation of critical
147 period plasticity that could lead to novel treatment targets for memory disorders and traumatic brain
148 injury.
149

150 # Materials and Methods

151 **Subjects**

152 A total of 42 adult Syrian hamsters (Cricetidae: *Mesocricetus auratus*) of both sexes were bred within
153 our animal facility and used as subjects in this study. Syrian hamsters are an altricial rodent species that
154 is ideal for studying the developing visual system due to their robust and well-characterized visual
155 responses, short gestation time, and large litters (Chalupa, 1981; Huck et al., 1988; Pratt and Lisk, 1989;
156 Razak et al., 2003; Carrasco et al., 2005). Sexual maturity in this species occurs between postnatal day
157 (P) 56 and P60 (Diamond and Yanagimachi, 1970; Fitzgerald and Zucker, 1976). Breeding females were
158 singly housed. Male breeders were introduced and supervised until intromission was observed, after
159 which they were removed. Weanlings and adult males were housed in single sex social groups of up to 5
160 adults per cage and adult females were housed with female siblings or individually in standard rodent
161 cages. Running wheels were not provided because they have been shown to alter the timing of visual
162 cortical plasticity (Baroncelli et al., 2010; Tognini et al., 2012; Kalogeraki et al., 2014) but a variety of
163 other enrichment items were available. All subjects were provided with *ad libitum* access to rodent chow
164 and water.
165
166 **Treatment groups**
167 Animals used in this study were bred in-house to control sensory experiences throughout development.
168 Dams of DR subjects were transferred into total darkness 1-3 days before parturition. An antechamber
169 and light-impenetrable black curtain separated the dark housing room from the hallway, ensuring that any
170 accidental openings of the hallway doors did not expose the animals to light. Dark reared animals were
171 housed inside light-tight stackable cages with a standard HVAC filtration system consistent with the other
172 animal rooms in the facility. During general animal husbandry purposes, the hamsters were exposed to
173 dim red light at a wavelength not visible to Syrian hamsters (Huhman and Albers, 1994). NR hamsters
174 were maintained in a standard 12/12 light cycle room from before birth into adulthood.
175
176 **Western blotting**

177 Animals were euthanized with Euthasol at >150 mg/kg IP. Brains were immediately extracted and frozen
178 in 2-methylbutane on dry ice, then stored at -80°C or immediately dissected for preparation of lysates.
179 Protein extraction was done as described by Shi et al. (1997) with a few modifications. Briefly, individual
180 left and right superficial SC brain areas were excised and homogenized in a lysis buffer (10 mM
181 phosphate buffer, pH 7.0, 5 mM EGTA, 5 mM EDTA, 1 mM DTT) containing Halt protease inhibitor
182 (ThermoFisher Scientific). The lysate was centrifuged at 16,000 × g at 4°C for 10 min, and the pellet was
183 resuspended in 2 mM HEPES buffer, pH 7.2. It was then centrifuged at 70,000 rpm at 4°C for 30 min.
184 The pellet thus obtained was resuspended in 0.5 mM HEPES, pH 7.3, containing 0.32 M sucrose and
185 centrifuged at 450 × g for 8 min. Synaptic proteins were quantified using the Pierce BCA Protein Assay
186 Kit (ThermoFisher Scientific), mixed with 2X sample buffer, and boiled for 15 min at 60 °C. Twenty µg
187 of protein was loaded per well in pre-cast Bio-Rad gels and electrophoresis was carried out at 110 V for
188 90 min in a Bio-Rad electrophoresis tank. Proteins were then transferred onto nitrocellulose membranes at
189 70 V for 75 min, blocked in BSA for 1 h, and probed with primary antibodies overnight. Primary
190 antibodies used in this study included: rabbit anti-$GABA_A$Rα1 (1:1000, Cat#: AGA-001, Alomone Labs);
191 rabbit anti- $GABA_A$Rα2 (1:1000, Cat#: ab72445, Abcam); rabbit anti- $GABA_A$Rα5 (1:1000, Cat#:
192 ab10098, Abcam); rabbit anti-Gephyrin (1:1000, Cat#: ab32206, Abcam); mouse anti-PSD-95 (1:1000,
193 Cat#: ab2723, Abcam); mouse anti-KCC2 (1:1000, Cat#: 75-013, NeuroMab); rabbit anti-NKCC1
194 (1:1000, Cat#: ab59791, Abcam); and mouse anti-GAPDH (1:1000, Cat#: 600-GAPDH,
195 PhosphoSolutions). Protein bands were labeled using either appropriate IR dye fluorescent secondaries or
196 appropriate HRP-conjugated secondary antibodies and imaged on an Odyssey CLx fluorescent imaging
197 system (Li-Cor) or developed with enhanced chemiluminescent (ECL) substrate in a Bio-Rad ChemiDoc
198 Imager. Protein expression levels in the bands were quantified as the optical density of the labeled protein
199 normalized against the optical density of the housekeeping protein GAPDH using ImageJ (NIH).
200
201 **Statistical Analysis**

202 A Student's *t*-test was used to compare parametric data with equal variance between treatment groups and
203 a normally distributed control data set. In the case of non-parametric data that were not normally
204 distributed and/or exhibited unequal variance, a Mann-Whitney Rank Sum (U) test was employed.
205 Descriptive statistics for these analyses are provided as mean ± standard error of the mean (SEM) in the
206 text.
207

208 # Results

209 The failure to maintain RF refinement in adult DR animals involves deficits in overall GABA
210 expression and $GABA_A$ receptor function (Carrasco et al., 2011), leading to a loss of lateral inhibition and
211 thus expansion of RFs after P60 (Carrasco et al., 2005; Balmer and Pallas, 2015b; Mudd et al., 2019). In
212 this study we explored several possible ways that visual deprivation during a critical period for RF
213 refinement could affect $GABA_A$ receptor efficacy and other postsynaptic proteins associated with
214 inhibitory synaptic function in adult SC. We used Western blotting on synaptosomes to study synaptic
215 proteins that can regulate synaptic plasticity but observed no significant changes in their relative
216 abundance under dark rearing conditions.
217

218 **Dark rearing does not affect the subunit composition of $GABA_A$ receptors in adult SC**

219 Deprivation-induced decreases in both $GABA_A$ and NMDA receptor levels in cat visual cortex have
220 been reported previously and were proposed to be involved in functional deficits (Carmignoto and Vicini,
221 1992; Chen et al., 2000; Chen et al., 2001). In DR hamsters, GAD and GABA immunoreactivity declines
222 and $GABA_A$ receptors are less efficient when tested pharmacologically (Carrasco et al., 2011), thus we
223 expected to see changes in levels of $GABA_A$ proteins postsynaptically. However, in our previous study
224 using Western blots on synaptosomes, no significant changes in the level of the $GABA_A$ receptor alpha1
225 subunit were observed (Mudd et al., 2019). These results raised the question of whether subunit

226 composition of $GABA_A$Rs might be altered by visual deprivation in a way that reduced their effectiveness
227 without affecting alpha1 levels.

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

244 $GABA_A$ receptors can also be expressed extrasynaptically, where they can be activated by ambient
245 GABA derived from synaptic spillover or non-neuronal sources. This low concentration GABA source
246 generates "tonic" inhibition (Farrant and Nusser, 2005), and constitutes a significant (~75%) portion of
247 the total inhibitory forces acting on neurons in hippocampus (Mody and Pearce, 2004; Magnin et al.,
248 2019). Alpha5 subunit-containing receptors are primarily expressed extrasynaptically and have been
249 implicated in regulating the induction of synaptic plasticity for LTP in hippocampus (Saab et al., 2010;
250 Zurek et al., 2012; Zurek et al., 2014; Jacob, 2019). However, alpha5 $GABA_A$Rs have also been shown to
251 relocate to the synapse, colocalize with gephyrin (Brady and Jacob, 2015), and are critical for memory

252 consolidation (Davenport et al., 2021). To investigate the possible role of alpha5 levels in adult RF
253 maintenance we performed Western blotting on synaptosomes and compared between NR (0.544 units ±
254 0.0520, n=8) and DR (0.471 ± 0.0935, n=8) adult hamsters **(Figure 3A)**. We found no significant
255 differences in alpha5 protein levels between groups (t (20) = 0.308, p = 0.878, Mann-Whitney Rank Sum
256 test. We also compared the ratio of alpha1/alpha5 between NR (1.027 ± 0.0815, n=10) and DR (0.995 ±
257 0.0926, n =9) adult hamsters and found no differences between these groups (U (18) =35, p=0.438, Mann-
258 Whitney Rank Sum test) **(Figure 3B)**. Because we were only studying proteins from synaptosome
259 preparations (i.e., $GABA_A$ receptor subunits localized in the synapses), these results suggest that the
260 localization of alpha1 and alpha5 subunit-containing $GABA_A$ receptors in SC is not being altered by early
261 visual experience and argue against a deprivation-induced shift in tonic vs. synaptic inhibition (Davenport
262 et al., 2021).
263
264 **Dark rearing does not affect the normal endocytosis of $GABA_A$ receptors in adult SC**
265 The regulation of $GABA_A$ receptors at the synapse is pivotal for maintaining correct levels of
266 inhibitory synaptic transmission (Jacob et al., 2008). Impaired trafficking of $GABA_A$ receptors into and
267 out of 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. Endocytosed
270 receptors are subsequently reinserted into the postsynaptic membrane or undergo lysosomal degradation
271 after longer periods (Kittler et al., 2000). We reasoned that if endocytosis was dysregulated, either by
272 decreased receptor reinsertion or increased receptor degradation, it could negatively impact the efficacy of
273 $GABA_A$ receptors at the synapse. We examined this possibility by comparing the ratio of postsynaptic
274 membrane-bound to cytosolic alpha1 subunit containing receptors between our treatment groups. No
275 differences were observed in the postsynaptic membrane/cytosol ratio of alpha1 expressing receptors
276 between DR (0.768 units ± 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 4A)**. These results indicate that the overall trafficking of
278 alpha1 (synaptic) subunit- expressing $GABA_A$ receptors is not affected by dark rearing.
279 We also examined the possibility that extrasynaptic alpha5 receptor endocytosis-mediated trafficking
280 may be dysregulated and thus responsible for changes in tonic $GABA_A$ inhibition (Davenport et al.,
281 2021). There were no significant differences in cytosolic membrane localization of alpha5 subunits
282 between adult DR (1.320 ± 0.198, n=8) and NR groups (1.753 ± 0.449, n=6) (U(12)=19.00, p=0.573,
283 Mann-Whitney Rank Sum test) **(Figure 4B)**. These results indicate that the overall exo- and endocytosis-
284 mediated trafficking of alpha5 (extrasynaptic) subunit-expressing $GABA_A$ receptors is not responsible for
285 the decreased efficacy of $GABA_A$ receptors observed in RFs that fail to maintain refinement in adulthood
286 after dark rearing.
287
288 **Inhibitory and excitatory scaffolding proteins in SC are not affected by dark rearing**
289 One factor influencing the accumulation and retention of $GABA_A$ receptors at postsynaptic sites is the
290 membrane scaffolding protein gephyrin (Kneussel et al., 1999; Sun et al., 2004; Jacob et al., 2005; Tretter
291 et al., 2008). Decreased expression of gephyrin results in less clustering (Essrich et al., 1998) and more
292 overall mobility of $GABA_A$ receptors at the synapse (Jacob et al., 2005). We surmised that decreased
293 gephyrin expression could be responsible for the weaker $GABA_A$ receptor signaling we observed in
294 neurons with RFs that fail to maintain refinement in adulthood. We quantified and compared postsynaptic
295 membrane-bound gephyrin expression between DR and NR adults using Western blotting. Gephyrin
296 levels in DR adults (0.786 ± 0.124, n=17) were similar to those in NR adults (0.736 ± 0.158, n=16)
297 (U(111)=-0.247, p=0.377, Mann-Whitney Rank Sum test) **(Figure 5A)**. This indicates that maintenance
298 of adult gephyrin expression levels is not affected by dark rearing and suggests that if $GABA_A$ receptor
299 accumulation and trafficking is being affected, then it is occurring in a gephyrin-independent manner.
300 PSD-95 is the primary glutamate (AMPA and NMDA) receptor scaffolding protein in CNS neurons
301 (Chen et al., 2015), and it functions in a similar way as gephyrin does for $GABA_A$ receptors. Although it
302 does not have a direct impact on $GABA_A$ receptor function, PSD-95 has been shown to have an influence

303 on visual plasticity. For example, mice lacking PSD-95 have lifelong ocular dominance plasticity in V1
304 that results from an increase in the overall proportion of silent synapses, despite having normal inhibitory
305 tone (Funahashi et al., 2013; Huang et al., 2015). The possibility that the plasticity involved with the dark
306 rearing-induced re-enlargement of RFs could be mediated by a reduction in adult PSD-95 expression was
307 examined. We found that PSD-95 protein levels were not significantly different in DR (0.613± 0.96,
308 n=10) compared to NR adult hamsters (0.486 ± 0.868, n=9) (U(31)=-0.978, p=0.270, Mann-Whitney
309 Rank Sum test) **(Figure 5B)**. These results suggest that differences in PSD-95 levels do not underlie the
310 re-enlargement of RFs in SC following dark rearing from birth.
311
312 **Cation-chloride co-transporters undergo their normal developmental switch in adult dark reared**
313 **subjects**
314 Levels of inhibitory GABAergic signaling in neurons are dependent on the intracellular chloride (Cl^-)
315 concentration. The K^+ Cl^- co-transporter (KCC2) is responsible for regulating intracellular Cl^- in mature
316 adult neurons by an outward K^+ current (Rivera et al., 1999) and also regulates the formation, function,
317 and plasticity of glutamatergic synapses (Li et al., 2007; Gauvain et al., 2011; Chevy et al., 2015). At the
318 beginning of postnatal life $GABA_A$ receptor effects are excitatory because the Na^+-K^+-$2Cl^-$ co-transporter
319 1 (NKCC1) that mediates Cl^- uptake is dominant (Cherubini et al., 1991; Lee et al., 2005; Cancedda et
320 al., 2007). By the end of the second postnatal week after birth in rats and mice NKCC1 is replaced by
321 KCC2 as the dominant cation-chloride co-transporter in the brain, shifting $GABA_A$ receptors to produce
322 inhibitory PSPs (Rivera et al., 1999; Pfeffer et al., 2009; Moore et al., 2019). In V1, the developmental
323 switch from NKCC1 dominance to KCC2 dominance occurs at the same time as a period of BDNF/TrkB
324 mediated synaptic imbalance (Zhang et al., 2018). We surmised that a shift in the ratio of KCC2:NKCC1
325 could underlie the reinstating/restoring of plasticity for RF size refinement in dark reared adults, leading
326 to re-enlargement of RFs in SC. Examination of the expression of KCC2 and NKCC1 in adult SC
327 neurons revealed no significant differences between our treatment groups, however. KCC2 levels were
328 not significantly different between NR (0.979 ± 0.115, n=8) and DR groups of adult hamsters (0.963 ±

329 0.154, n=8) (T(14)=0.082, p=0.936, t-test) **(Figure 6A)**. The same was true of NKCC1 levels (NR 1.050
330 ± 0.0419, n=8; DR 1.081 ± 0.0814, n=8) (T(14)=-0.339, p=0.740, t-test) **(Figure 6B)**, and of the ratio of
331 the two cation-chloride co-transporters within groups (T(8)=1.096, p=0.305, t-test) **(Figure 6C)**.
332

333 **Discussion**

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

348 This study supports our previous research that provided substantial evidence of a novel,
349 maladaptive adult plasticity in hamsters, in which visually deprived hamsters develop refined RFs
350 normally but fail to maintain them in adulthood. Interestingly, this contrasts with previous literature on
351 other mammals including cats (Cynader and Mitchell, 1980; Carmignoto and Vicini, 1992; Chen et al.,
352 2000; Chen et al., 2001; Mower, 1991; Mower et al., 1985; Mower et al., 1986; Erisir and Harris, 2003),
353 and rats (Fagiolini et al., 1994; Lee and Nedivi, 2002), in which the visual system does not develop
354 normally in response to dark rearing. Some of these previous studies looked only at early and/or adult

355 ages in the animals, thereby missing the RF refinement that happens in between the two ages. Because
356 diminished GABA release, contrary to what we expected, did not elicit changes in the levels of major
357 postsynaptic $GABA_A$ receptors, scaffold proteins, or chloride co-transporters, this study provides a
358 valuable demonstration that changes in neurotransmitter availability do not necessarily result in
359 coordinated changes in postsynaptic receptors.

360 Maturation of GABAergic signaling in visual cortex, particularly of the fast-spiking, parvalbumin-
361 containing basket cells, is thought to open and then close the critical period for plasticity (Fagiolini et al.,
362 2004; Sale et al., 2010; Toyoizumi et al., 2013; Capogna et al., 2021). Combined pre- and postsynaptic
363 alteration of synaptic strength has been seen in other sensory deprivation paradigms, including in dark
364 reared and monocularly deprived visual cortex, although with an earlier time course (Carmignoto and
365 Vicini, 1992; Chen et al., 2000; Chen et al., 2001). However, the retinorecipient layers of the superior
366 colliculus have no basket cells and contain very few GABAergic parvalbumin neurons, and the plasticity
367 described here occurs after the critical period should have closed, suggesting that SC may accomplish
368 plasticity through a different mechanism than visual cortex. On the other hand, previous studies found
369 that, as in visual cortex (Hanover et al., 1999; Huang et al., 1999; Viegi et al., 2002), deprivation-induced
370 receptive field plasticity in adult SC is mediated by the BDNF receptor TrkB (Mudd et al., 2019).
371 Furthermore, reduced GABA and $GABA_A$ receptor efficacy in response to iontophoretically-applied
372 $GABA_AR$ agonists and antagonists is observed in both SC and V1 of visually deprived hamsters
373 (Carrasco et al., 2011; Balmer and Pallas, 2015b), arguing for mechanistic elements in common.
374

375 ***Early visual experience is not necessary for maturation or maintenance of $GABA_A$ receptor subunit***
376 ***composition at the synapse***

377 $GABA_A$ receptors contain fast acting chloride (Cl^-) channels (Pfeiffer et al., 1982; Sigel and
378 Steinmann, 2012). The subunit composition of $GABA_A$ receptors changes during development from an
379 alpha 2 to alpha 1 dominant condition (Laurie et al., 1992; Fritschy et al., 1994; Chen et al., 2001) and
380 also changes in some disease states (Levitt, 2005; Tyson and Anderson, 2014; Deidda et al., 2015;

381 Kimoto et al., 2015; Schmidt and Mirnics, 2015; Tang et al., 2021) in a way that affects receptor
382 functional properties (Farrant and Nusser, 2005) and localization (Jacob et al., 2005). We studied
383 synaptic levels of the $GABA_A$ receptor alpha1 and alpha2 subunits to quantify their expression levels
384 under normal and DR conditions. The overall levels of $GABA_AR$ alpha1 and $GABA_AR$ alpha2 were not
385 altered in DR hamsters when compared to those of NR hamsters, arguing that the altered inhibitory
386 synaptic efficacy that we previously observed was not caused by an immature $GABA_AR$ subunit
387 composition at the synapse.

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

395

396 ***Level and localization of the extrasynaptic $GABA_AR$ subunit alpha 5 does not change with visual***
397 ***deprivation***

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

407

408 ***Visual deprivation does not affect the rate of endocytosis of $GABA_AR$ subunits at the synapse***

409 Because clathrin-dependent endocytosis is likely important for regulating inhibitory signaling and
410 synaptic plasticity (Kittler et al., 2000), we examined the endocytosis of $GABA_A$ receptor alpha1 and
411 alpha5 subunits in normally reared and visually deprived subjects. We did not observe a significant
412 change in endocytosis of either subunit type as assayed by the ratio of synaptosome-bound to cytosolic
413 fractions. This implies that visual deprivation does not affect the trafficking of the $GABA_AR$ subunits
414 between the synaptic membrane and the cytosol or the phosphorylation events that maintain the balance
415 between endocytosis and postsynaptic membrane 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 early visual deprivation, suggesting that any
420 changes in inhibitory function are probably not caused by a significant change in scaffolding protein
421 expression. Alternatively, the clustering of $GABA_ARs$ at inhibitory synapses in SC may happen in a
422 gephyrin-independent manner (Zita et al., 2007), or total gephyrin expression may not be as important as
423 the formation of gephyrin nanodomains within inhibitory synapses (Pennacchietti et al., 2017). Future
424 studies with different techniques would be required to determine if changes in receptor clustering may be
425 occurring and to what extent 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
429 maintaining chloride balance inside the neurons and thus in maintaining the E/I balance. The cation-
430 chloride co-transporters could have reverted to their early developmental state, leading to disturbed E/I
431 balance and more excitation in dark reared animals, possibly explaining the RF expansion we observed.
432 However, we did not observe any changes in the cation-chloride co-transporters in dark-reared compared

433 to normally reared adult hamsters, suggesting that the changes in E/I balance due to lack of early visual
434 experience were not caused by alterations in the cation-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 synaptic release of GABA (Huang et al., 1999; Morales et al., 2002; Gianfranceschi et al.,
443 2003; Jovanovic et al., 2004; Kuczewski et al., 2008; Porcher et al., 2011; Hanno-Iijima et al., 2015),
444 maintaining RF size and visual acuity through GABAergic lateral inhibition (Mudd et al., 2019).
445 Signaling via the MAPK cascade and the transcription factor cAMP-responsive element-binding protein
446 (CREB) appears to play a substantial role in this process (Obrietan et al., 2002). BDNF-TrkB interaction
447 leads to dimerization and auto-phosphorylation of TrkB, thereby triggering MAPK, PLC gamma, and
448 PI3K pathways (Yoshii and Constantine-Paton, 2007). These pathways in turn lead to the activation of
449 downstream effectors and mediators to initiate a CREB-dependent transcription process that can lead to
450 an increase in $GABA_A$R levels as well as more BDNF production (Huang and Reichardt, 2003; Yoshii
451 and Constantine-Paton, 2007; Porcher et al., 2011; Esvald et al., 2020). In addition, an increase in the
452 transmembrane localization of $GABA_A$Rs is mediated by BDNF-dependent inhibition of receptor
453 endocytosis in addition to ongoing reinsertion of the receptor into the postsynaptic membrane (Porcher et
454 al., 2011). This positive feedback regulation is critical in developing neurons and hence constituted a
455 major motivation for the work reported here. In this study however, neither the $GABA_A$R subunit
456 composition at the synapse nor subunit composition in the extrasynaptic regions was affected by dark
457 rearing. Chloride transport proteins (KCC2, NKCC2) also remained normal in levels. One possibility is
458 that GABA expression levels alone are the key factor in RF re-enlargement (Carrasco et al., 2011; Mudd

459 et al., 2019), suggesting that this type of delayed plasticity resulting from a lack of early visual experience
460 occurs through a different mechanism than described in other types of plasticity resulting from visual
461 deprivation.

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

470 Suggested alternative explanations to understand this form of adult neural plasticity include that
471 the changes leading to RF expansion and thus the visual acuity deficits take place earlier than the time
472 point that we studied and return to normal by P90. Previous research has shown that the width of the
473 synaptic cleft decreases during development (Li and Cline, 2010). Thus, another interesting possibility is
474 that dark rearing from birth gradually increases the width of the synaptic cleft again in adulthood, while
475 maintaining the postsynaptic signaling components in place. It is also possible that other scaffolding
476 proteins and their partners are involved, for example gephyrin binding partners such as $GABA_AR$ beta2
477 and beta3 subunits (Kowalczyk et al., 2013), the scaffold protein radixin that binds $GABA_AR$ alpha5
478 subunit to the actin cytoskeleton (Loebrich et al., 2006) and regulates synaptic $GABA_AR$ density (Hausrat
479 et al., 2015), or gephyrin post-translational modification events that influence inhibitory synaptic
480 plasticity by affecting postsynaptic scaffolding (Zacchi et al., 2014). More recently, distinct spectrin
481 isoforms have been shown to affect synaptic inhibition by selectively targeting specific $GABA_ARs$
482 subtypes to dendritic and axon initial segment synapses (Smalley et al., 2023).

483 In summary, our results argue that visual experience is not necessary to maintain mature levels and
484 composition of several postsynaptic proteins that are essential for retino-SC synaptic communication.

485 Unlike many previous visual deprivation studies in which both GABA and $GABA_A$ receptors are
486 downregulated (Carmignoto and Vicini, 1992; Chen et al., 2000; Chen et al., 2001; Kilman et al., 2002;
487 Nahmani and Turrigiano, 2014), we report here that in SC $GABA_AR$ levels, subunit composition, and
488 localization in adulthood are unaffected by dark rearing. The scaffold proteins gephyrin and PSD-95, and
489 the chloride transporters KCC2 and NKCC2 are also not affected by a lack of light exposure. The novel,
490 experience-dependent form of adult plasticity that we observed previously may involve an as yet
491 unidentified postsynaptic mechanism or solely a reduction in GABA release (Carrasco et al., 2005;
492 Carrasco and Pallas, 2006; Carrasco et al., 2011), thereby challenging the common argument that
493 presynaptic changes in ligand availability are always associated with matching postsynaptic changes in
494 their receptors. Either possibility is encouraging with respect to understanding this form of adult plasticity
495 and could help to improve treatment and therapeutic modalities in adults with memory impairments,
496 traumatic brain injury, or inhibition-associated neurological disorders.
497

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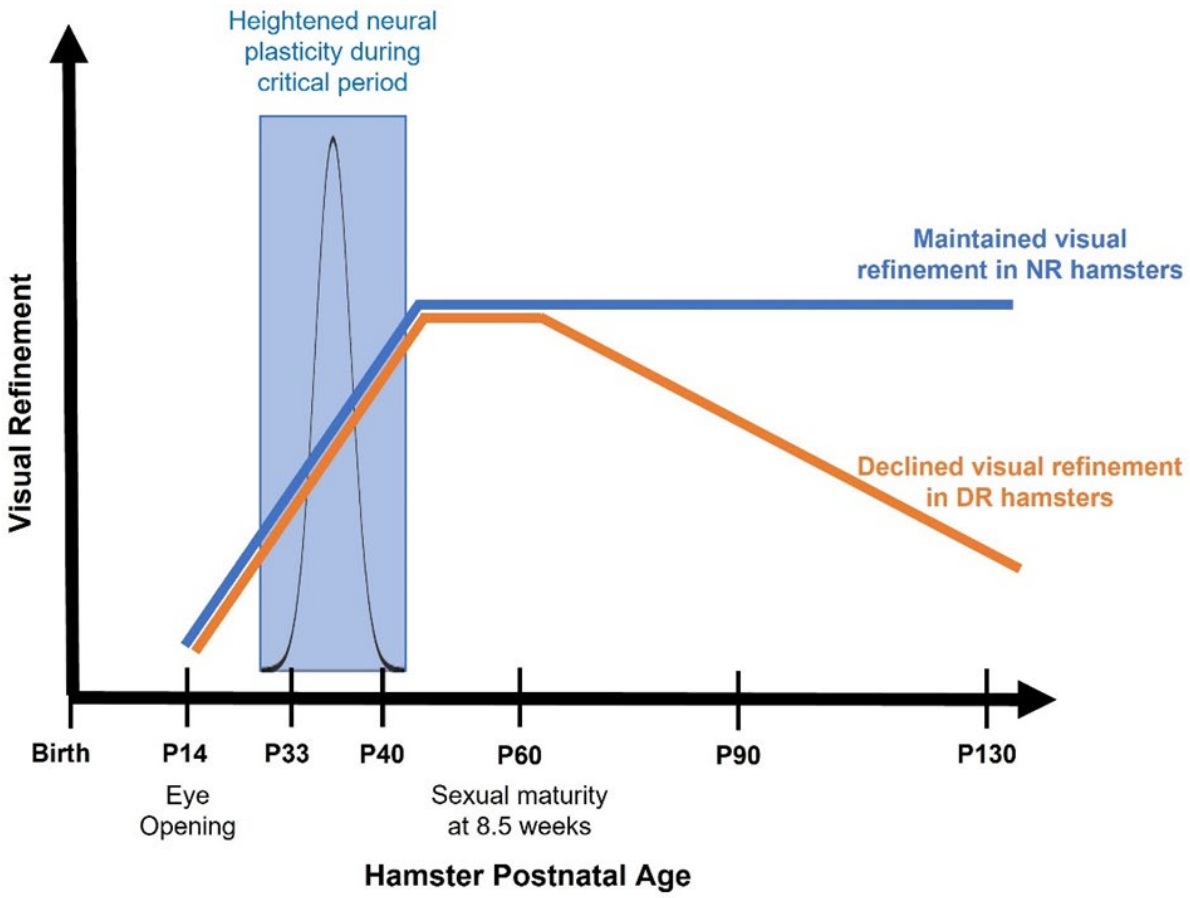
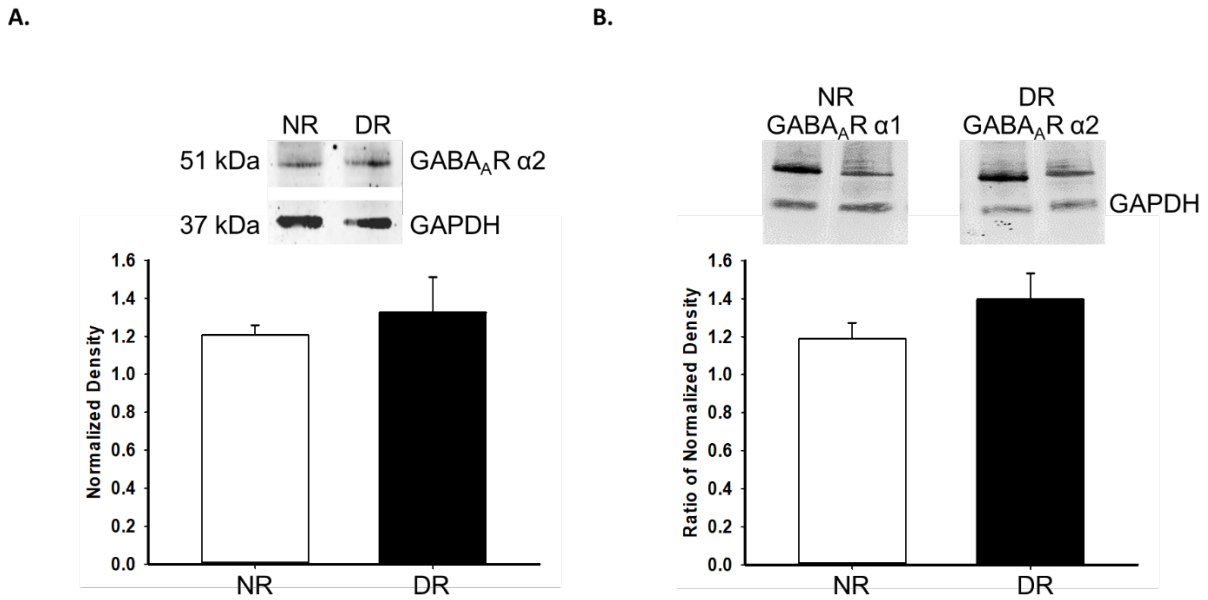


Figure 1: Differences in the visual refinement in the hamster SC depending on exposure to light during the critical period of heightened neural plasticity. Hamsters exposed to light during their critical period gradually improve RF refinement in the SC and maintain it throughout life (as indicated by the blue line). Hamsters that do not experience light during the critical period also show RF refinement in the SC by P60, but the refinement progressively declines in adulthood (as indicated by the orange line) (Carrasco et al., 2005; Carrasco and Pallas, 2006; Balmer and Pallas, 2015b).



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797 **Figure 2. Adult GABA$_A$α2 receptor subunit composition is not affected by early dark rearing. (A)**
798 image: Individual Western blots of normally reared (NR) and dark-reared (DR) treatment groups
799 generated using 20 µg of SC protein per lane. GAPDH was used as a loading control. graph:
800 Densitometric analyses of GABA$_A$Rα2 in normally vs. dark reared hamsters. **(B)** Comparison of
801 GABA$_A$Rα2 expression with GABAA$_A$Rα1 expression, shown as individual Western blots in the upper
802 panel and as a ratio in the lower panel. Data presented as mean ± SEM. In this and the following figures,
803 lanes presented together are from the same gel(s), and each measured protein was normalized against
804 GAPDH as a loading control. These results suggest that the number and ratio of synaptic GABA$_A$α2
805 receptors are similar in normal and dark reared adult SC.

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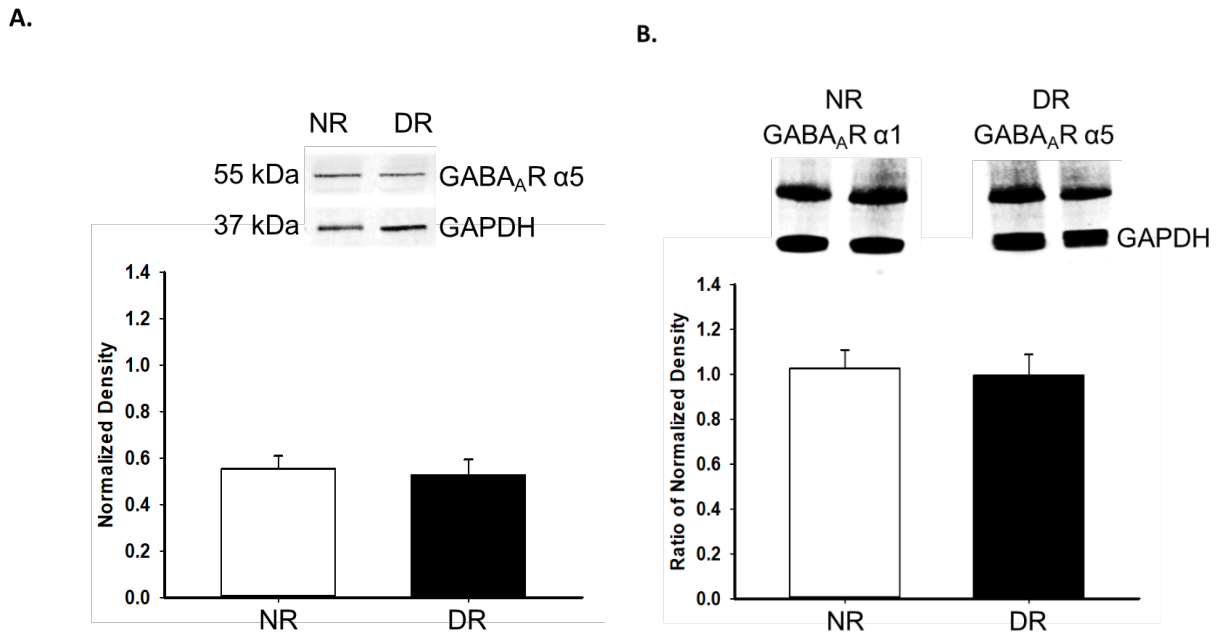

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810 **Figure 3. Adult GABA$_A$α5 receptor subunit composition is not affected by early dark rearing. (A**

811 **and B top:** Example Western blots of NR and DR treatment groups as in Figure 2. All presented lanes are

812 from the same gel(s), and each gel was run with GAPDH as a loading control. **(A graph)** GABA$_A$Rα5

813 comparison. **(B graph)** GABA$_A$Rα1: GABA$_A$Rα5 ratio. Data presented as mean ± SEM normalized

814 against GAPDH levels. These results suggest that the number and ratio of synaptic GABA$_A$α5 receptors

815 are similar in normal and dark reared adult SC.

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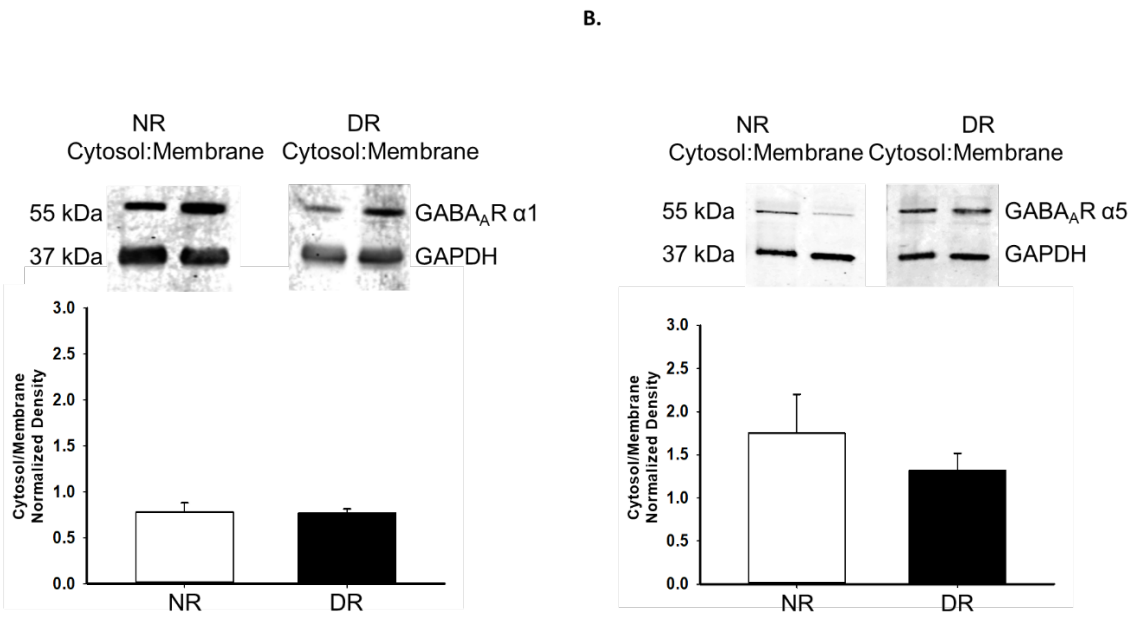


Figure 4. Endocytosis of GABA$_A$ receptors is not affected by early dark rearing. (A) Adult levels of the cytosolic vs. synaptic membrane-attached ratio of the extrasynaptically-targeted GABA$_A$Rα1 and **(B)** the synaptically-targeted GABA$_A$Rα5 subunits were not affected by early light deprivation. Example Western blots (upper panels) represent bands of labeled intracellular and membrane bound receptor subunit proteins (upper bands) from the same animal, measured as a ratio against GAPDH and compared between NR and DR groups. GAPDH was used as a loading control. Graph data presented as normalized mean optical density ± SEM.

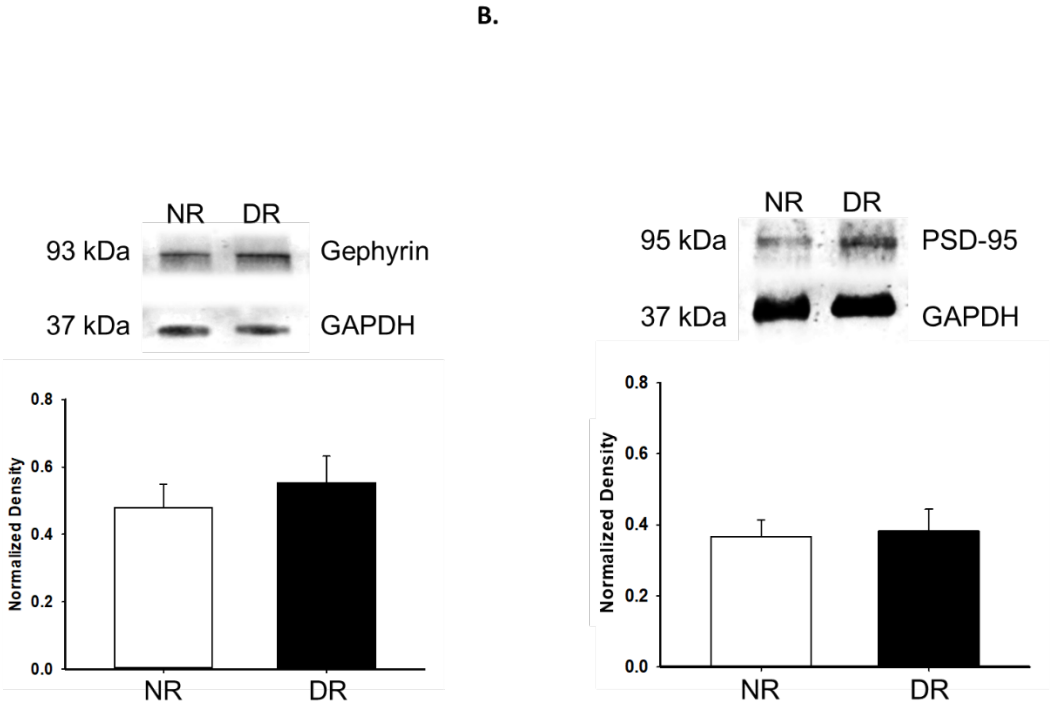
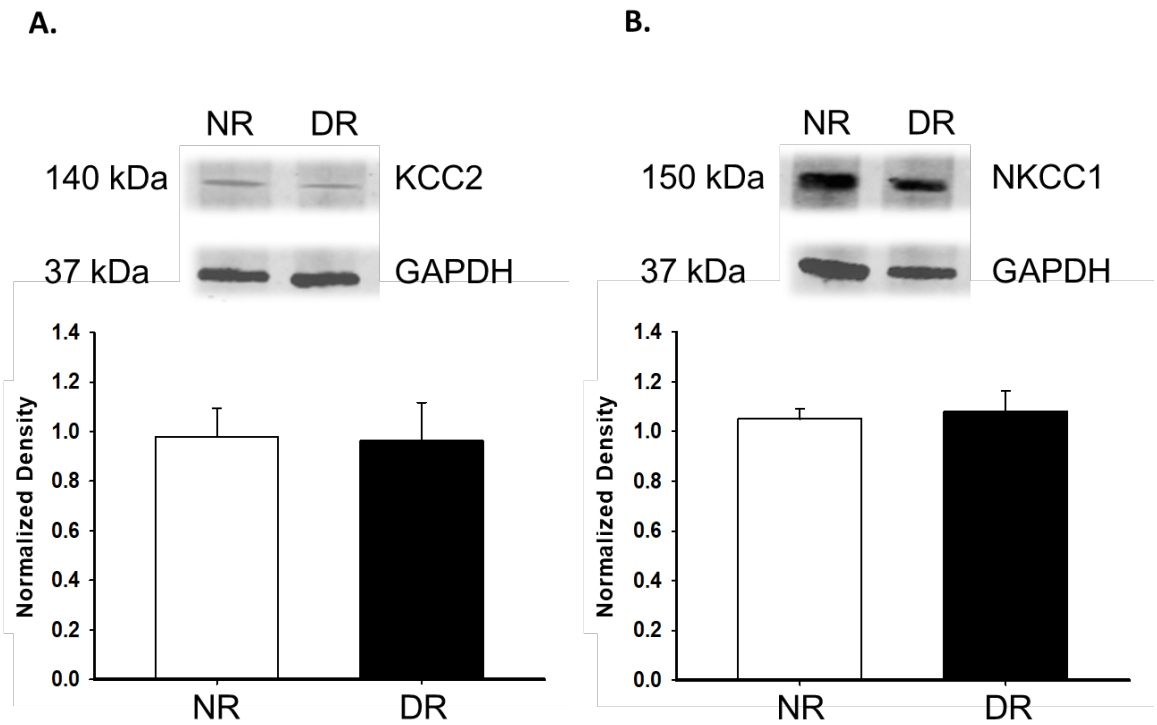


Figure 5. Adult gephyrin and PSD-95 expression are not affected by dark rearing. (A) Gephyrin and (B) PSD-95 expression was similar between adult (>P90) NR and DR groups. GAPDH and β-actin were used as loading controls. Graph data are presented as normalized mean relative optical density ± SEM.



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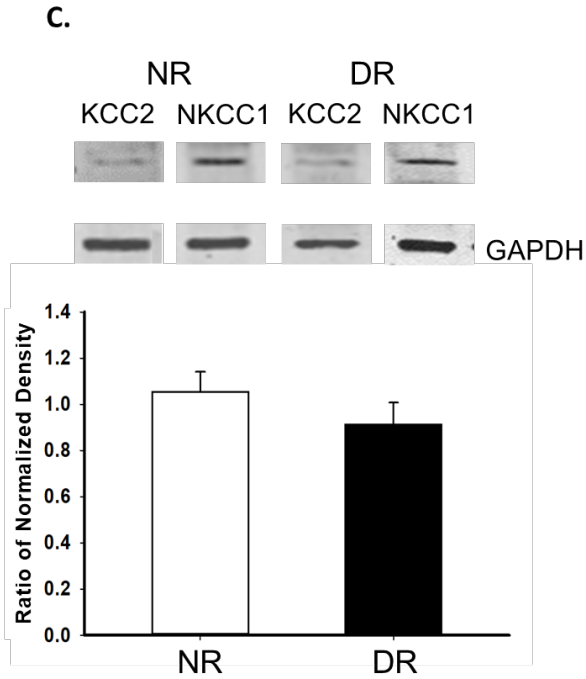

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838 **Figure 6. Adult cation-chloride co-transporters expression is not affected by early dark rearing.**

839 Western blot examples of NR and DR samples labeled for cation-chloride co-transporters **(A)** KCC2 (140

840 kDa) and **(B)** NKCC1 (150 kDa), **(C)** and a comparison of the within subject ratio of KCC2:NKCC1 in

841 NR and DR adult (>P90) hamsters. Graph data presented as mean relative optical density ± SEM.

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