Functional genomic mechanisms of opioid action and opioid use disorder: a systematic review of animal models and human studies

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Abstract

In the past two decades, over-prescription of opioids for pain management has driven a steep increase in opioid use disorder (OUD) and death by overdose, exerting a dramatic toll on western countries. OUD is a chronic relapsing disease associated with a lifetime struggle to control drug consumption, suggesting that opioids trigger long-lasting brain adaptations, notably through functional genomic and epigenomic mechanisms. Current understanding of these processes, however, remain scarce, and have not been previously reviewed systematically. To do so, the goal of the present work was to synthesize current knowledge on genome-wide transcriptomic and epigenetic mechanisms of opioid action, in primate and rodent species. Using a prospectively registered methodology, comprehensive literature searches were completed in PubMed, Embase, and Web of Science. Of the 2705 articles identified, 69 met our inclusion criteria and were considered for qualitative analysis. Focusing on the 5 most studied nervous system structures (nucleus accumbens, frontal cortex, whole striatum, dorsal striatum, spinal cord; 44 articles), we also conducted a quantitative analysis of differentially expressed genes, in an effort to identify a putative core transcriptional signature of opioids. Only one gene, Cdkn1a, was consistently identified in eleven studies, and globally, our results unveil surprisingly low consistency across published work, even when considering most recent single-cell approaches. Analysis of putative sources of variability detected significant contributions from species, brain structure, duration of opioid exposure, strain, time-point of analysis, and batch effects, but not type of opioid. To go beyond those limitations, we leveraged threshold-free methods to illustrate how genome-wide comparisons may generate new findings and hypotheses. Finally, we discuss current methodological development in the field, and their implication for future research and, ultimately, better care.

Keywords

Opioid, substance use disorder, genomics, epigenomics, next generation sequencing

Introduction

Opioids have been used for millennia for their analgesic and euphoric properties[1]. While they remain reference pain treatments, their chronic use also associates with tolerance, physical dependence and, in some individuals, the emergence of an opioid use disorder (OUD). OUD is defined as a problematic use leading to significant impairment. This severe and chronic disorder associates with a life expectancy decreased by more than 10 years due to somatic and psychiatric comorbidities. Over the last 2 decades, western countries have faced an increase in death by opioid overdose, due to more frequent prescription for pain management, increasing use of illegal compounds, or misuse of substitution therapies. OUD imposes a major socio-economic burden, with an estimated annual cost in the trillion dollar range in the US[2], and increasing use and harm in Europe[3]. This results, overall, in a worsening public health crisis.

OUD results from a variety of interacting biological, psychological and socio-economic factors[4]. At biological level, it originates from pharmacological effects of opioid drugs, which trigger chemical and molecular brain adaptations, under modulation by genetic vulnerability and epigenetic regulation. In turn, these effects mediate behavioral and cognitive dysfunctions, including the inability to control drug use despite harmful consequences, or persistent and intense desire for the drug, even after years of abstinence.

Since the 1990s, high-throughput approaches have been harnessed to characterize the molecular underpinnings of opioid effects in brain tissue, throughout the full genome[5], across various experimental models and species. In this context, the goal of the present systematic review was to synthesize current knowledge on functional genomic mechanisms of opioid action and OUD, defined as changes in gene expression or epigenetic regulation. To do so, we used a preregistered methodology, and performed an unbiased survey of bibliographic repositories, focusing on high-throughput studies, most notably microarrays and next-generation sequencing. While we acknowledge the important contribution of candidate gene studies (already reviewed in the past, see[6, 7]), this focus on genome-wide analyses

reflects the conviction that the understanding of heterogeneous phenotypes such as OUD may benefit from analyzing the full genome.

Based on studies identified through this screening, a qualitative synthesis was first performed to summarize experimental designs and findings. Second, a focused quantitative analysis of bulk tissue transcriptomic studies was conducted, with the goal of defining a core signature of opioids. To our knowledge, while numerous reviews are regularly published in the opioid field[8–12], such a systematic synthesis across brain structures and technologies had never been performed. Our results indicate that, although more than 40 genome-wide studies have been published, available evidence for convergent findings is surprisingly limited. Third, we reviewed most recent work that leveraged cell-type specific and single-cell approaches, or that interrogated epigenetic regulatory mechanisms contributing to opioid plasticity. Finally, we discuss current challenges in the field, as well as avenues and recommendations for future work.

Methods

Full methodology and code are available as Supplementary Material and File.

Results

The identification and selection of eligible studies is presented in Fig.1. For qualitative synthesis, 69 papers were selected, covering both transcriptomic and epigenomic approaches. Among these, the 44 articles that performed transcriptomic analyses in the 5 most frequently investigated regions were selected for more detailed quantitative synthesis: of these, 34 investigated a single brain region, 9 covered 2 regions, and only 1 characterized 5 regions[13]. This resulted in 52 differential expression analyses: 18, 11, 11, 6 and 6 in the nucleus accumbens (NAc), frontal cortex, whole striatum, dorsal striatum and spinal cord, respectively.

1. Qualitative analysis (n=69 studies)

107 ***Species and sex ratio.*** Most studies were conducted in rodents (Fig.2A): 30 (43%) in rats,
108 28 (41%) in mice, and 1 in both species. Only a small number investigated humans (n=8, 12%)
109 and macaques (n=2, 3%), reflecting well-known practical limitations. As consequence, current
110 knowledge on conserved or species-specific opioid effects is limited.

111 Regarding sex, epidemiological surveys indicate that OUD prevalence is higher in
112 males than in females[3, 14] (although smaller differences have been reported with DSM-5
113 criteria[15]), with a number of sex-specific risk factors (e.g., ethnicity, income). This clinical
114 situation has long been modeled in rodents, as sex differences in behavioral effects of
115 opioids[16] have been described for analgesia[17], tolerance[18], withdrawal symptoms[19],
116 reward[20] or motivation[21]. In comparison, molecular mechanisms underlying these
117 differences are poorly characterized. The majority of OUD studies reviewed here investigated
118 males only (n=48, 70%; Fig.2B-D), with few conducted in females (n=5, 7%), and only 14
119 (20%) that included both sexes (7 in rodents, 7 in humans). These numbers are consistent
120 with the current landscape of neuroscience research, in which the ratio of articles reporting on
121 males only, as opposed to females only, is around 5 to 1[22]. We nevertheless note an
122 encouraging trend, as the large majority of studies published in 2021 (8/10) investigated both
123 sexes. Several factors likely underlie this evolution: policies from funding agencies (such as
124 the NIH; https://grants.nih.gov/grants/guide/notice-files/not-od-15-102.html); increased
125 recognition that females do not display more experimental variability than males[23]; and
126 evidence that pathophysiological mechanisms may differ across sexes, as shown for
127 depression[24], schizophrenia[25] or alcohol use disorder[26].

128 Specifically in rodents (Fig.2C), 80% of studies investigated males only (n=47). Seven
129 articles investigated both sexes (n=7, 12%), using surprisingly low sample sizes (n=1-4 in
130 male/female, and opioid-treated/control, groups; Fig.2D). Among these, 2 studies treated sex
131 as a covariate[27, 28], and 3 pooled both sexes for the analysis of opioid effects[29–31],
132 therefore providing no direct description of male/female differences. This is concerning,
133 considering that the 2 remaining studies found little overlap, from 5 to 35% (see[32] and[33]),
134 among morphine-induced changes occurring in each sex. Therefore, available evidence

135 points toward substantial sex-specificity in opioid-induced transcriptomic plasticity, which will
136 have to be confirmed in future work. In human, even less is known (Fig.2E). Among 8 articles,
137 7 investigated males and females, while only 1 investigated males only. However, these 7
138 studies showed a strong bias towards examination of men (Fig.2F), with only one that used
139 equal sample sizes in both sexes[34]. In the latter, sex was treated as a covariate, with no
140 specific description of its impact. Altogether, this significant gap in the literature calls for
141 additional work, with the hope that better understanding of sex-specific pathophysiological
142 routes may unravel more efficient therapeutic strategies.
143
144 ***Route and duration of opioid administration.*** Among studies included in qualitative analysis
145 (n=69), the majority used chronic administration (n=55, 80%; see Fig.3A), mostly via self-
146 administration (SA, n=19, 34%, including 8 human studies) and intraperitoneal injection (ip;
147 n=18, 33%), followed by subcutaneous injection (n=8, 15%). Other procedures were less
148 frequently used: continuous administration (with pellets or mini-pumps; n=5, 9%), intrathecal
149 administration (n=3, 5%), or gavage (n=2, 4%).
150 There is limited information regarding differences among administration procedures.
151 Interestingly, Lefevre et al[35] recently compared 3 conditions: continuous and systemic
152 morphine infusion using osmotic mini-pumps, with ("Interrupted") or without ("Continuous") the
153 induction, twice daily, of a precipitated withdrawal episode (using naloxone, a non-specific
154 opioid antagonist), compared to Controls (saline mini-pumps). Results indicated 687 and 407
155 differentially expressed genes (DEG) when comparing Interrupted and Control groups in the
156 NAc and dorsal striatum, respectively, with only 1 DEG between Continuous and Control
157 groups. Therefore, rather than opioid exposure per se, it is the repetition of intermittent
158 withdrawal episodes that may drive the intensity of transcriptomic adaptations affecting the
159 mesolimbic system (whether precipitated, as in this study, or spontaneous, in-between bolus
160 injections). This is consistent with the clinical notion that the alternation of intoxication and
161 withdrawal phases is critical for the development of OUD.

162 Opioid effects have also been investigated using acute administration (10 studies,
163 14%), corresponding mostly to ip injection (9 articles), with only 1 using subcutaneous
164 injection. While these studies were mainly interested in the use of opioids as analgesics or
165 anesthetics[36, 37], or sought to compare acute effects of various psychoactive drugs[38],
166 their results may be relevant for the understanding of OUD. Indeed, comparisons of inbred
167 mouse strains found striking variability in the intensity of opioid physical dependence, with the
168 same strains showing severe or mild signs of withdrawal when precipitated after acute or
169 chronic opioid injections[39, 40]. In other words, there may be shared genetic determinants,
170 within each strain, for brain adaptations triggered by acute or chronic exposure. A recent
171 single-cell RNA-sequencing study (scRNA-Seq; see *3. High cellular resolution approaches*
172 and [41]) provides an interesting illustration of how such strain-specific genetic traits may
173 translate into at least partially similar transcriptional plasticity during acute or chronic morphine
174 exposure.

175 Another important consideration is that most studies (51/69, 73%) used passive
176 administration, which do not model volitional or compulsive drug-taking. This reflects the
177 technical difficulty of intravenous SA experiments (IVSA), particularly in the mouse. Few
178 studies have conducted direct comparison of passive and active administrations. Among
179 these, Tapocik et al[42] compared “yoker” mice (IVSA group) with groups of “yoked-morphine”
180 and “yoked-saline” animals which, each time the yoker self-administered morphine, received
181 passive IV morphine or saline injections. Results significantly differed depending on the mouse
182 strain used. In C57BL/6J, a much higher number of DEG were identified between the yoker
183 and yoked-saline groups (n=1176) than between yoked-morphine and yoked-saline mice
184 (n=244), with limited overlap between the 2 (n=103). In DBA/2J mice that did not self-
185 administer morphine (no behavioral differences among the 3 same groups), transcriptional
186 effects associated with operant responding were milder than in C57BL/6J (n=107 DEG
187 between yoker and yoked-saline groups), as expected, while again a modest number of DEG
188 between yoked-morphine and yoked-saline groups (n=262, likely corresponding to
189 pharmacological morphine response). This suggests that distinct transcriptional adaptations,

190 partly dependent on genetic background, may underlie voluntary drug-taking or reflect non-
191 contingent passive pharmacological effects, and together contribute to OUD.
192 New paradigms have been developed to facilitate such studies, which notably include
193 oral SA[43–45]), or devices for opioid vapor SA[46]. In addition, optogenetic tools now enable
194 light-induced self-stimulation (optogenetic intracranial self-stimulation, oICSS)[47]. The latter
195 models are based on selective manipulation, using opsins, of the activity of anatomically- or
196 genetically-defined neuronal populations. While these approaches do not recapitulate the
197 systemic effects of opioid IVSA, they are nevertheless able to recapitulate similar behavioral
198 deficits, including uncontrolled consumption, resistance to punishment, or excessive
199 motivation[48, 49]. It is therefore possible that they may allow dissecting the neuronal
200 pathways that are necessary or sufficient for each of these behavioral dimensions, as well as
201 underlying molecular mechanisms. Another avenue for improvement relates to the mode of
202 action of genetic tools used for oICSS. Upon light-stimulation, opsins open ion channels that
203 modulate neuronal excitability. Their high temporal resolution does not necessarily represent
204 the best model for the longer time-frame of pharmacological action of drugs of abuse. To more
205 faithfully mimic such kinetics and associated intracellular signaling, chemogenetics or chimeric
206 opsins (for light-induced metabotropic signaling, with a proof-of-concept available for
207 MOR[50]) might represent better tools.
208
209 ***Nervous system structures.*** Among articles eligible for qualitative analysis, 18 regions of the
210 nervous system were explored (Fig.2B). Not surprisingly, a large majority focused on the
211 mesocorticolimbic dopaminergic pathway: the NAc (21 articles[13, 27, 30, 33–35, 37, 51–64]),
212 frontal cortex (13 ; [13, 29, 32, 34, 62, 65–72]), whole/unspecified striatal complex (11 ; [31,
213 38, 73–81]), dorsal striatum (7 ; [13, 35, 59–61, 82, 83]) and ventral tegmental area (VTA, 5 ;
214 [33, 62, 84–86]). Other regions included the spinal cord (7 ; [73, 87–92]); hippocampus (5 ;
215 [13, 74, 93–95]); amygdala (3 ; [13, 96, 97]); locus coeruleus[85, 98], ventral midbrain[42, 99],
216 hypothalamus[100, 101] (2 each), whole brain with[102] or without[103] cerebellum,
217 periaqueductal gray matter[104], pituitary gland[101], arcuate nucleus[105], nucleus of the

218 tractus solitarius[36], brainstem[28], cerebellum[95] or dorsal root ganglia[106] (DRG, 1 each).
219 Below, we briefly describe the rationale for studying such diverse structures.

220 The course of OUD involves 3 stages: intoxication, withdrawal/negative affect, and
221 anticipation of next intoxication. These stages reflect gradual adaptation of the brain to drug
222 exposure, and rely on distinct mechanisms. The intoxication stage, mostly driven by acute
223 reward, notably associates with dopamine release, in the striatum and frontal cortex, by
224 neurons located in the VTA. The second stage corresponds to negative reinforcement,
225 whereby drug consumption alleviates physical and negative affective states of withdrawal,
226 involving the locus coeruleus[107], NAc[108], and amygdala[109], among others. Finally,
227 anticipation results from memories of drug-associated cues and contexts (hippocampus), as
228 well as impaired goal-directed behaviors, implicating higher-order structures (e.g. prefrontal
229 and orbital cortices[110]). These relationships are not exclusive, with some structures
230 implicated at multiple stages (e.g., the striatum, also involved in habit formation). Other regions
231 (spinal cord, DRG, brainstem, periaqueductal gray matter, PAG) were investigated in relation
232 to opioid analgesia, tolerance and hyperalgesia. Finally, few articles were interested in how
233 opioids modulate neuroendocrine systems (hypothalamus, arcuate nucleus, pituitary gland).

234 In addition to these associations, it is also important to consider the distribution of the
235 endogenous opioid system. This system is composed of 3 opioid receptors (mu, delta and
236 kappa; MOR, DOR and KOR), among which the MOR is necessary for opioid-induced
237 analgesia, reward, and physical dependence[111]. Neurons expressing this receptor therefore
238 represent pharmacological entry points for brain adaptations to opioids. Comparison of studies
239 reviewed here with MOR brain distribution (assessed using ligand autoradiography[112–117];
240 Fig.3C) indicates that: i) MOR is highly expressed among structures most frequently studied,
241 as expected; ii) surprisingly, other areas with similarly high expression have received little to
242 no attention (NTS, locus coeruleus, dorsal raphe nucleus, medial habenula, thalamus), calling
243 for more work.
244

245 ***Techniques.*** Thirty-seven and 29 studies used microarrays and RNA-seq, respectively, to
246 analyze the transcriptome (Fig.3D). While the first RNA-seq study was published in 2014[37],
247 this technique has since progressively replaced microarrays, reflecting well-recognized
248 advantages (higher sensitivity and throughput, gene discovery). For epigenomic profiling, 3
249 studies recently performed bisulfite sequencing[62, 70, 72], and 1 used ATACseq (Assay for
250 Transposase-Accessible Chromatin with sequencing)[83]. Considering rapid progress in
251 sequencing methodologies (see below), these trends are expected to strengthen.
252

253 **2. Quantitative analysis of transcriptomics studies**

254 To identify reproducible transcriptomic opioid signatures, we ran quantitative analyses of bulk-
255 tissue results available for the 5 most studied structures (44 articles; Fig.4A). To do so, gene-
256 level results were retrieved when available. This yielded 24 lists of DEG comparing opioid-
257 treated animals and control groups, originating from 17 articles. Among these, 6 articles did
258 not report any p-values, while the remaining 11 used very diverse thresholds on p-values and
259 fold changes (FC; Supplementary Table 1). Only a small minority (3/44, 6.8%[31, 33, 34])
260 provided p-values and FC for all genes. In addition, raw data were available in the public
261 repository GEO in less than half of studies reporting DEG (8/17, 47%). Overall, this
262 underscores strong heterogeneity in data analysis and reporting, which limits re-use[118]. To
263 counter this, efforts have been made to harmonize practices in the field[119, 120], with
264 guidelines enforced by funding agencies[121]. For the present review, a genome-wide meta-
265 analysis combining, for every gene, effect and sample sizes across studies was not possible.
266 We nevertheless: i) Systematically assessed concordance among available lists of DEG; ii)
267 Conducted a threshold-free comparison of the 2 NAc studies for which genome-wide results
268 were available.
269

270 **2.1 Nucleus accumbens**

271 Among 18 articles, only 8 provided lists of DEGs: 4 in mice (males only[35, 37, 51, 54]), 2 in
272 rats (1 in males[56], 1 in both sexes[33]), 2 in humans (both sexes[34, 58]). The 8 articles

273 provided a total of 3502 DEG (Fig.4B-E). Among these, the vast majority were identified by 1
274 study only (n=3017/3502, 86%), while 424, 57 and 4 DEG were identified across 2, 3 or 4
275 studies, respectively. No single genes were common to 5 or more articles. In addition, the best
276 overlap was found among the 2 DEG lists reported by Imperio et al[56] (Fig.3E). Overall, this
277 indicates low concordance among NAc studies.

278 To define factors contributing to this heterogeneity, we intersected DEG lists in each
279 species separately (Fig.3C-E). This, however, did not improve concordance: in humans, only
280 54 genes were common to both studies (Jaccard Index, JI=2.4%, Fig.4C, Supplementary
281 Table 2 for the matrix of pair-wise JI across all studies); in mice, 7 were identified by at least
282 2 studies (JI=1%, Fig. 4D); in rats, the JI increased to 15.5% (Fig.4E), mostly driven by high
283 overlap among the 2 aforementioned DEG lists from Imperio et al. In the latter work, the
284 authors identified large- (LS) and small-suppressors (SS): in LS rats, greater avoidance of a
285 natural reward (saccharine) that predicted heroin availability was associated, compared to SS
286 rats, with worse outcomes during later heroin IVSA (increased consumption, higher motivation
287 and relapse). Gene expression changes occurring in the LS group (compared to control rats
288 given access to saccharine but not heroin; n=597 genes), and in the SS group (same controls,
289 n=205) showed a JI of 25.5% (with striking concordance in up- and down-regulation). This
290 was much higher than overlap between Imperio et al and the other rat study, by Townsend et
291 al (Imperio-SS/Townsend, JI=1.35%; Imperio-LS/Townsend, 3.43%), suggesting that
292 differences across laboratories and animal facilities may significantly contribute to poor
293 concordance. Importantly, while the relatively small number of studies in the NAc precluded
294 definitive conclusions, a more powerful analysis of sources of variability was possible across
295 our 5 regions of interest (see section *2.6*).

296

297 **2.2 Dorsal striatum**

298 Only 2 dorsal striatum studies provided DEG (Fig.3F). One study used chronic morphine
299 administration interrupted by naloxone injections in male mice[35], as described above, while
300 the other used acute morphine injection in male rats[82]. Reflecting these differences, a very

301 small overlap was found between the 2 (0.62%). Therefore, while the dorsal striatum is thought
302 to gradually gain control over drug-taking in OUD[122], underlying transcriptomic plasticity is
303 poorly characterized.
304
305 **2.3 Whole striatum**
306 Six of the 11 whole striatum studies provided DEG[31, 38, 75–78] (Fig.5B): 2 investigated
307 chronic heroin[78] or morphine[31], while 2 compared acute effects of distinct opioids
308 (morphine and heroin)[38, 76], and 2 compared acute and chronic morphine[75, 77]. Most
309 DEG were identified by 1 study only (3308/3736, 88.5%), with only 112 genes common to 3
310 or more studies. Consistent with NAc results, strongest overlaps were observed for lists of
311 DEG coming from the same article, with for example 54 (JI=17%, Korostynski 2013[38]) and
312 16 (21%, Piechota 2010[76]) DEG common to acute morphine and heroin injections,
313 respectively, and 139 DEG (31%, Korostynski 2007 [77]; Fig.5A) common to acute and chronic
314 morphine administration. In sharp contrast, comparisons across, rather than within, studies
315 yielded poor overlaps, even when considering those that used similar drug administration:
316 among chronic morphine studies (Fig.S1A), the JI varied from 0.1 to 2.6% (Korostynski
317 2007/Skupio 2017), and, among acute morphine studies, from 2.7% to 7.8% (Fig.S1B), with
318 only one comparison standing out among acute heroin studies (30 common DEG, JI=19%,
319 Piechota 2010/Korostinsksy 2013). Importantly, 5 of these 6 studies in the whole striatum were
320 conducted by the same group, suggesting that significant variability may persist even among
321 studies conducted in the same facility, but at different periods (see also section 2.6).
322
323 **2.4 Frontal cortex**
324 Seven studies provided DEG (Fig.5B): 2 in humans[34, 69], 5 in rats[29, 32, 66–68]. Data from
325 Odegaard et al[29] were discarded, as they corresponded to transgenerational effects in
326 animals not directly exposed to opioids. For 2 additional studies [66, 68], we could not map
327 microarray probes to genes. Among the 4 remaining articles, little overlaps were again found
328 (Kuntz-Melcavage/Mendez, JI=0.5%; Kuntz-Melcavage/Seney, 1.1%; Liu/Mendez, 1.4%;

329 Liu/Seney, 1.6%), with no improvement within species (Liu/Kuntz-Melcavage in rats, JI=0%;
330 Seney/Mendez in humans, 4.1%).
331
332 **2.5 Spinal cord**
333 Among the 6 spinal cord studies, 5 did not provide DEG[87–91], while the 6th one used a
334 homemade microarray[73], for which correspondence between probe IDs and genes was only
335 partially provided. As such, no comparison was possible.
336
337 **2.6 Sources of variability**
338 Considering that pharmacological recruitment and intra-cellular signaling of MOR may trigger
339 a set of common transcriptional adaptations across the 5 regions of interest, we next pooled
340 all lists of DEGs (Fig.5D). Again, the vast majority of DEG (70.1%, 4940/7047) were unique to
341 a single study, while only 21.4% common to 2 studies (1509), 5.9% to 3 (418), and 1.7% to 4
342 or more (117; Supplementary Table 3). Interestingly, Cdkn1a (Cyclin Dependent Kinase
343 Inhibitor 1A) was identified by 11 studies. This gene encodes for p21, a protein involved in the
344 regulation of the cell-cycle and oligodendrocytes[123]. Interestingly, Avey et al[51] found
345 Cdkn1a to be one of the most significantly upregulated genes in response to acute morphine.
346 This upregulation occurred in oligodendrocytes, and was blocked by the opioid antagonist
347 naltrexone, indicating a MOR-dependent mechanism. Of note, cocaine, another major drug of
348 abuse, similarly recruits p21, as cocaine-induced behavioral responses were modified in p21
349 knockout mice[124]. Using this knock-out line, or virally-mediated approaches for p21
350 manipulation, represent appealing perspectives to investigate this gene in OUD.
351 We also performed Gene Ontology (GO) analysis using DEG common to at least 3, 4
352 or 5 studies (Supplementary Table 4). No results were significant for the small list of 63 DEG
353 common to 5 or more studies. When considering the 180 DEGs common to at least 4 studies,
354 only 3 GO terms were enriched: cell periphery, plasma membrane and hormone activity.
355 Results more directly related to nervous system function emerged for the 598 DEG common
356 to at least 3 studies, with 46 significant GO terms, mostly related to synaptic signaling and

357 cell-cell communication, likely reflecting opioid-induced electrophysiological and synaptic
358 adaptations across brain structures.

359 Because weak DEG overlaps were identified within individual regions (even when
360 grouping studies based on species, Fig.4C-E, or treatment chronicity, Fig.S1), we sought to
361 more systematically assess, across the 5 regions, the impact of following factors: species,
362 brain structure, treatment chronicity, drug, strain, time-point of assessment following last
363 exposure, and article (Fig.5D). For each factor (e.g., species), pair-wise JI were classified in
364 2 categories depending on whether they belonged to the same sub-group within that factor (2
365 studies in humans; “intra”), or not (1 human study compared to another in rats; “inter”). As
366 expected, grouping list of DEG by species (p=2.5E-3), brain structures (3.4E-8), treatment
367 chronicity (p=1.4E-3), mouse genetic background (3.9E-5) or the time-point of analysis after
368 opioid exposure (6.7E-10) all increased concordance, with higher JI. This is consistent with
369 the notion that, although they remain poorly characterized, these 5 variables contribute to
370 opioid-induced transcriptional plasticity. In contrast, the type of opioid used had none
371 (p=0.059) when considering the 13 and 5 rodent DEG lists related to morphine or heroin,
372 respectively (human studies were discarded, as the types of opioids consumed are diverse
373 and/or poorly characterized). This is surprising, considering that these 2 MOR agonists, known
374 to exhibit strong pharmacokinetic differences (heroin being a lipophilic prodrug that more
375 rapidly crosses the blood brain barrier to be metabolized in 6-monoacetylmorphine and
376 morphine, which activate MOR), may be expected to generate distinct transcriptomic
377 adaptations. Time-course and dose-response experiments may therefore be necessary to
378 detect such potential differences. Importantly, concordance increased most when DEG lists
379 came from the same publication (p=2.5E-5), despite the fact that only 7 comparisons fell under
380 this category. As already mentioned, this suggests that differences across laboratories may
381 explain a substantial part of the lack of concordance across studies.

382 Finally, we refined our analysis by considering the directionality (up/down) of DEG,
383 focusing on genes identified by at least 5 studies (n=63). Among results from 17 out of 24
384 studies that reported on directionality (Fig.S2), most genes (37/63, 59%) showed discordant

385 results (while, for 8 others, directionality was reported by only 1 or no study). Only 18 genes
386 showed 100% concordance across studies (29%; 17 up-regulated, 1 down), among which 11
387 were identified by at least 3 studies. The latter included the aforementioned Cdkn1a, as well
388 as Slc2a1, Fkbp5, Sult1a1, Arrdc3, Ccdc117, Plin4, Wscd1, Arid5b, Pla2g3, and Tsc22d3.
389 Slc2a1 encodes for the glutamate transporter GLT1, which plays a major role in extracellular
390 glutamate uptake and the regulation of excitatory transmission. A recent study showed that
391 during prolonged morphine withdrawal, activation of KOR in the amygdala leads to increased
392 GLT1 expression and excitatory drive in the NAc, thereby mediating withdrawal-induced
393 depressive-like behaviors. Another interesting hit is Fkbp5, a chaperone and intra-cellular
394 negative regulator of glucocorticoid signaling, that has been largely investigated for its role in
395 stress-related disorders[125], including OUD[126]. The 8 remaining genes, although
396 comparatively less studied in relation to opioids (Fig.S2), represent additional candidates for
397 future work.
398
399 **2.7 Threshold-free genome-wide comparison**
400 Next, we compared the 2 NAc studies (Seney et al[34], Townsend et al[33]) for which full
401 genome-wide results were available. While both included males and females, only Townsend
402 reported sex-specific results. Therefore, we reprocessed Seney's raw data to identify DEG in
403 each sex separately (*Supplementary Material*). This slightly increased concordance: while JI
404 were equal to 7.5 and 4% when comparing initial Seney data (male/females pooled) with
405 Townsend data in females or males, respectively (Fig.S3A-B), they increased to 9.5% and
406 4.5% when considering Seney's sex-specific results (Fig.6A-B). This argues for the
407 importance of accounting for sex.
408 We then used RRHO2 (Rank–Rank Hypergeometric Overlap[127]) to compare the 2
409 studies. RRHO2 iteratively performs hypergeometric testing for all combinations of thresholds
410 applied to each dataset, generating a "threshold-free" analysis. Because this approach takes
411 directionality into account, we also computed, for comparison, overlaps among either up- or
412 down-regulated DEG reported by each study (white crosses, Fig.6C-D). Compared to those,

413 much more significant overlaps were identified when considering less stringent p-value
414 thresholds with RRHO2 (best hypergeometric results shown as black crosses). By definition,
415 these increased overlaps, came at the expense of the identification of large gene lists which,
416 although commonly dysregulated in similar direction, individually exhibited milder FC.
417 Interestingly, these overlaps were detected despite the fact that Seney investigated human
418 post mortem tissue from individuals with OUD, while Townsend focused on rat tissue collected
419 after 24 hours of withdrawal from fentanyl IVSA. Also, different patterns of convergence were
420 observed in each sex: in females, overlaps were highly significant among both up- and down-
421 regulated genes while, in males, a lower concordance predominantly affected down-regulated
422 genes. This suggests that the rat dataset may differentially recapitulate part of the sex-specific
423 signatures of human OUD. Overall, these analyses illustrate the utility of reporting full genome-
424 wide results which, in combination with threshold-free approaches, generate deeper
425 comparisons (see *Discussion*).
426

427 **3. High cellular resolution approaches**

428 Studies reviewed above indicate that opioids induce genome-wide transcriptomic changes,
429 with striking variability across laboratories, experimental designs, and brain regions. This likely
430 reflects a combination of: i) direct pharmacological opioid effects, corresponding to the
431 recruitment of the MOR intracellular signaling, and resulting in decreased neuronal
432 excitability[128]; ii) indirect effects: depending on the chemical identity and connectivity pattern
433 of MOR-expressing cells, their inhibition leads to secondary changes affecting other
434 neurotransmitters[113]. This raises 2 questions: how is each cell type, or even each brain cell,
435 affected by these changes? What are underlying molecular regulatory principles?

436 To address the first question, several groups recently used cell-type specific and
437 single-cell methodologies. Coffey et al[31] focused on microglial cells of the whole striatum,
438 using translational ribosome affinity purification (TRAP, to capture ribosomes and the
439 "translating transcriptome"[129, 130]). Chronic morphine effects were analyzed with, or
440 without, the induction of naloxone-precipitated withdrawal. Results showed that both chronic

441 morphine and withdrawal triggered numerous changes in gene expression, which mostly
442 affected GO terms related to cAMP signaling, regulation of synapses, and the cytoskeleton. A
443 striking negative correlation was observed between the 2 sets of results, providing a correlate,
444 at the level of the microglial transcriptome, of the pharmacological antagonism between
445 morphine and naloxone. Considering the inter-strain variability in the sensitivity to both acute
446 and chronic opioid physical dependence described earlier (see section *1.Qualitative analysis-*
447 *Route and duration of opioid administration* and [39, 40]), an interesting perspective would be
448 to determine whether naloxone similarly antagonizes transcriptomic effects of acute opioid
449 exposure, and how this is modulated by underlying genetic variation. Of note, surprisingly,
450 such cell-type specific approaches have not been used to interrogate opioid effects in neuronal
451 cells, let alone opioid-responsive MOR-expressing neurons which, arguably, may be
452 particularly sensitive. This is feasible, as knock-in lines in which the Cre recombinase is
453 selectively active in MOR-expressing cells are available[131–133].

454 Achieving higher resolution, Avey and colleagues applied single-cell RNA-seq
455 (scRNA-Seq, using Drop-seq[134]) to analyze transcriptomic changes induced by acute
456 morphine in more than 23,000 cells of the mouse NAc [51]. As is typical in scRNA-Seq,
457 sequencing depth yielded data for ~1,600 genes, corresponding to a reduced genomic
458 representation. Changes were widespread and, interestingly, most frequent in
459 oligodendrocytes, followed by neurons, astrocytes, and microglia, therefore uncovering a
460 cellular hierarchy in transcriptional sensitivity to opioids. More recently, Reiner et al[41] used
461 single-nucleus RNA-Seq (snRNA-seq, 10x Genomics 3') to analyze a larger number of nuclei
462 (190,030) and genes (>2000) in the rat NAc, also following acute morphine passive injection.
463 Compared to the former study, DEG mostly affected neurons and oligodendrocytes, followed
464 by astrocytes and microglia. Ten days of morphine IVSA was also investigated, and compared
465 to acute effects. Among DEG shared across the 2 conditions (n=431), the vast majority were
466 dysregulated in a similar direction (up/down), suggesting that they may reflect morphine
467 exposure rather than volitional drug-taking (a "yoked" group chronically exposed to morphine
468 would help substantiate this distinction). Importantly, a larger number of DEG were specifically

469 dysregulated in only one condition (n=868, with more DEG associated with IVSA), with distinct
470 distributions among NAc cell-types (e.g., Dopamine receptor D1- or D2-expressing neurons).
471 By providing the first single-cell description of morphine IVSA, these results reinforce the
472 notion that voluntary drug-intake may recruit partly distinct molecular mechanisms from those
473 of non-contingent administration.

474 We next compared results on acute morphine from these 2 studies. Both used
475 Seurat[135] and dimensionality reduction (t-SNE[51] or UMAP[41]) to identify clusters, which
476 were then assigned to cell-types using external data. We first compared marker genes
477 reported to define those clusters, when available (Avey: 11/29 clusters; Reiner: 11/19
478 clusters). Low agreement was found (Fig.S4A), with JI ranging from 0 up to 7.1% (for Avey's
479 activated medium spiny neurons, MSNs, and Reiner's D1R MSN-1), possibly because both
480 studies reported low numbers of marker genes (Avey: 39-51; Reiner: 4-12). We then
481 confronted morphine-induced DEG for each cell-type (Fig.6E). Low JI were found, with
482 maximal overlap (6.4%) obtained when comparing DEG affecting Avey's "myelin-forming and
483 mature oligodendrocytes" (MO_all) with Reiner's "Oligodendrocyte-3". Surprisingly, while
484 some clusters assigned to similar cell-types in the 2 studies showed some similarity in DEG
485 (green squares, Fig.6E), strong concordance was also observed across clusters with different
486 identities (e.g. Reiner's "Astrocyte-2" and Avey's MO_all; yellow squares). Overlaps among
487 DEG (Fig.6E) did not increase as a function of cell-type similarity across studies (Fig.S4B), as
488 would be expected. While particular emphasis has been put in sc/snRNA-Seq studies on the
489 number of individual cells to be sequenced, these results suggest that in order to maximize
490 their utility: i) marker genes used to assign cellular identities to clusters should be reported
491 comprehensively; and ii) rather than short lists of DEG, differential expression results should
492 be provided for all genes analyzed in each cluster. In addition, classical issues related to
493 sample size, power or statistical models for group comparisons remain pregnant. The Avey
494 and Reiner studies used similar sample sizes (4 and 5 replicates per opioid or control group,
495 respectively), in the upper range of bulk tissue studies (Fig.2D), but applied distinct tools to
496 identify DEG (the SCDE and MAST packages, respectively), which may partly account for

497 discrepancies. With the rapid evolution of the sc/snRNA-Seq field, and emerging controversies
498 (e.g. on dimensionality reduction[136]), additional efforts will be necessary to reach consensus
499 on designs and analyses[137, 138].
500

501 **4. Non-coding and epigenomic mechanisms**

502 In parallel to efforts to deepen cellular resolution, others have sought to decipher underlying
503 non-coding RNA and epigenomic regulations[139]. Although neurons are post-mitotic cells,
504 they exhibit peculiar plasticity features that may contribute to the encoding of life events,
505 including exposure to psychoactive drugs[140]. Several non-coding RNA species have been
506 investigated. The first report used custom-made arrays to characterize micro-RNA (miR) and
507 identify bidirectional expression changes affecting 7 of them, in rat primary neuronal cultures
508 or the mouse hippocampus, following morphine or heroin exposure[95]. Among these, miR-
509 339-3p was shown to target the 3'-UTR and down-regulate MOR in response to opioid
510 treatment[141]. A second study used more recent arrays to interrogate all mouse miR in the
511 NAc, following chronic heroin injections[63]. 111 miR exhibited >25% expression changes (no
512 significance reported). The study focused on miR-218, and showed that its over-expression
513 inhibited heroin-induced reinforcement (in conditioned place preference and IVSA), possibly
514 by targeting MeCP2 (a protein that binds methylated DNA), suggesting a potential link
515 between opioids, miR and DNA methylation. Other studies explored long non-coding (lncRNA)
516 or circular RNA (circRNA). One group used 2 arrays interrogating 9,000 lncRNA[89] and
517 15,000 circRNA[92] to characterize chronic morphine effects in the rat spinal cord: 136 lncRNA
518 (84 up, 52 down; nominal p-value<0.05, |log2FC|>1.5) and 2038 circRNA (896 up, 1142 down;
519 nominal p-value<0.05, FC>2) were affected. Another report used RNA-Sequencing to focus
520 on circRNA in the NAc[64], and found differences for 112 of them when comparing mice
521 receiving chronic morphine injections with or without electroacupuncture (51 up, 61 down;
522 nominal p-value<0.05, |log2FC|>1.5). Results from these studies remain descriptive, and
523 numerous mechanisms have been proposed to explain how non-coding RNA may exert
524 biological functions (including the sequestering of miR by circRNA, or complementarity

525 interactions among lncRNA and mRNA). Understanding how these mechanisms are recruited
526 by opioids will require further work.

527 Regarding the epigenome, 3 recent studies (1 in rats[62], 2 in humans[142, 143])
528 investigated DNA methylation[144]. The rat study used Whole Genome Bisulfite Sequencing
529 (WGBS) to analyze DNA methylation changes after heroin IVSA, in a standard or enriched
530 environment, in 3 brain regions (VTA, NAc and medial prefrontal cortex)[62]. Because of low
531 sequencing depth, DNA methylation levels were averaged across the whole genome for
532 selected features (promoters, CG islands). As expected, neither IVSA nor environmental
533 enrichment had any impact on these metrics, suggesting that the methylome is not massively
534 reprogrammed. Opioids may still, however, trigger more discrete DNA methylation changes,
535 a hypothesis supported by: i) the intuition that the large transcriptomic changes associated
536 with opioid exposure or OUD reviewed above are unlikely to occur in the absence of any
537 chromatin plasticity; ii) candidate studies which, although outside our scope, provide evidence
538 for differential DNA methylation at specific loci (the MOR gene in human blood[145–147]; few
539 loci in the rat brain[148]); and iii) results from the 2 human studies. Focusing on Brodmann
540 area 9 (BA9), the first applied EPIC arrays to a post-mortem cohort of OUD patients (n=19)
541 and healthy controls (n=11)[143]. Among >800,000 CG sites (where DNA methylation mostly
542 occurs in the mammalian brain), 11,917 located at transcription start sites (TSS) showed
543 nominally significant differences (p-value<0.05), with bidirectional changes (unfortunately,
544 genomic localization of these probes, and full genome-wide results, were not provided).

545 Such human studies face the difficult task of accounting for confounding factors.
546 Variation in genotype, age, sex, or socio-demographic factors, for example, have all been
547 associated with DNA methylation changes. Also, because epigenetic landscapes determine
548 cellular identity, group differences observed in bulk tissue may reflect variations in cellular
549 composition (possibly contributing to pathogenesis), rather than transcriptional regulation. The
550 second human study started to address these questions[142]. Using EPIC arrays to analyze
551 bulk tissue, the authors adjusted for socio-demographic characteristics, ancestry and cellular
552 composition. They investigated a large cohort (n=153), focusing on death from acute opioid

553 overdose. Although no individual CG site survived multiple testing correction, 13 passed a
554 relaxed significance threshold (p<1.0E-05), one of which was located in Netrin-1, a gene
555 implicated in KOR activity. At genome-wide level, opioid use associated with accelerated
556 epigenetic aging, along with enriched differential methylation in the KEGG pathway related to
557 dopaminergic signaling. Overall, these 2 human studies support the notion that the brain DNA
558 methylome may be modulated by opioids. More work will be necessary to better understand
559 these effects, as well as potential roles for non-canonical, brain-specific forms of DNA
560 methylation (methylation in non-CG context[149], hydroxymethylation at CG sites[150].

561 Beyond DNA modifications, epigenomic plasticity also relies on histone modifications
562 and changes in chromatin structure. Surprisingly, no data characterizing histone changes in
563 relation to opioid exposure were identified during our systematic screening. However, a recent
564 study compared chromatin accessibility in a cohort of 10 heroin users who died from heroin
565 overdose, and 10 healthy individuals[83], in the NAc and putamen. ATAC-Seq was used to
566 identify genomic regions where chromatin was "open", and DNA accessible. The technique
567 was applied to neuronal and non-neuronal cells, isolated using NeuN staining and
568 Fluorescence-Activated Nuclear Sorting (FANS). Heroin use associated with an enrichment
569 of ATAC-Seq peaks in specific gene features (CG islands, promoters, 5' untranslated regions)
570 and annotated regulatory regions (the histone mark H3K4me1, binding sites for the EZH2
571 transcription factor), as well as more frequent changes in neurons than in non-neuronal cells
572 (affecting GO terms related to the synapse, among others). At behavioral level, the authors
573 then showed that FYN (a tyrosine kinase with changes in chromatin accessibility in human)
574 significantly contributed to heroin IVSA in the rat. Overall, this work illustrates how epigenetic
575 analyses may uncover chromatin regulation contributing to OUD.

576

577 **Discussion**

578 Here we systematically assessed current knowledge on functional genomic mechanisms of
579 opioid action and OUD, and uncovered a surprisingly low convergence across studies. As a
580 result, a lot remains to be done to move the field forward. Below, we propose

581 recommendations for study design and data sharing, as well as perspectives to refine
582 behavioral modelling, improve cellular resolution, and advance multiomic data integration.
583
584 ***Recommendations for study design and data sharing.*** The low sample sizes observed in
585 the present review (Fig.2D,F) were surprising, considering that this factor is arguably the most
586 important one influencing statistical power[151–153]. Accordingly, a more rigorous analysis of
587 power, and larger sample sizes, will likely lead in future work to better reproducibility, and
588 should be enforced, especially when studying rodent outbred strains, or humans, who display
589 higher inter-individual variability[154, 155]. In humans, similar to what has been observed for
590 Genome-Wide Association Studies (GWAS[156]), functional genomic studies of OUD are
591 expected to evolve in the next decade towards collaborative efforts involving multiple labs (e.g.
592 the SCORCH consortium[157]), and the progressive aggregation of ever larger cohorts.
593 Another major recommendation relates to data sharing. While public repositories have been
594 specifically implemented for collecting and disseminating omic data (GEO[158], the IHEC data
595 portal[159]) or bioinformatic pipelines (Zenodo, GitHub), their systematic use is not yet current
596 practice in the OUD field, as exemplified in the present work (with similar criticisms raised in
597 other research domains[160]). This hampers re-analysis of published data, comparisons of
598 results across laboratories, and the use of threshold-free methods. We believe this last point
599 is particularly meaningful. Most opioid studies report subsets of DEG identified using arbitrary
600 significance cut-offs, which can mask subtle changes holding relevant biological information
601 (Fig.6[127]). We therefore strongly advocate for generalized use of threshold-free algorithms
602 for enrichment analysis of single datasets (GSEA), or comparison across pairs of differential
603 analysis results (RRHO2). Within this line, newer packages are expected to scale-up the
604 applicability of such tools to epigenomic data, which frequently involve larger number of
605 observations than gene-level counts[161].
606
607 ***Refine behavioral modeling.*** The face validity of SA or oICSS models of OUD has
608 significantly increased over last 2 decades [162], and we now face the task of more

609 systematically combining them with most recent molecular approaches. Another important
610 step will be to decipher the molecular mechanisms that may differentially account for distinct
611 behavioral dimensions, among impulsive or persistent drug-seeking, resistance to
612 punishment, or excessive motivation (including those modelled using passive administration,
613 such as tolerance, sensitization, or withdrawal). While the vast majority of studies reviewed
614 here used a categorical comparison of animals exposed or not to opioids, another design,
615 based on a dimensional approach within the exposed group, could help address the inter-
616 individual variability in susceptibility or resilience to OUD. The latter design has been used by
617 only one article in the present review[56] (section *2.1*), which contrasts with the amount of data
618 available for models of other psychiatric disorders, in particular depression (e.g. chronic social
619 defeat[163–165]. To better understand such inter-individual susceptibility, epigenetic
620 mechanisms are obvious candidates[166], as illustrated by 2 recent studies on DNA
621 methylation and oral SA of alcohol in macaques[167], and miRNA in a rat model of cocaine
622 SA[168]. While similar mechanisms are likely at play in OUD, they have not been addressed
623 by open-ended approaches yet, pointing toward an important avenue for future work.
624
625 ***Improve cellular resolution***. This is another major avenue for future work, considering the
626 extreme heterogeneity of brain tissue. In addition to cell-type specific (TRAP) and sc/snRNA-
627 Seq (already applied to OUD, see above), a newer generation of tools now enables similar
628 molecular profiling while taking neuroanatomical context into consideration, with 2 possible
629 factors: i) connectivity patterns, probed using antero- or retrograde viruses, in combination
630 with TRAP (transcriptomics[169]) or FANS (epigenomics[170]); or ii) spatial context, with
631 methodologies based on sequencing or microscopy to detect RNA “in histological space”;
632 while spatial capture followed by transcriptome sequencing (e.g. Slide-seq[171]) enables
633 genome-wide analysis with 10µm resolution, single-molecule fluorescence in situ hybridization
634 (smFISH) allows for better spatial resolution (1µm), but requires probe design and target gene
635 selection[146,147] (covering a few hundred to a few thousand genes, lower than sc/snRNA-
636 Seq[174]). Future work using these techniques, by characterizing molecular effects of opioids

637 in a particular neuronal pathway, or with exquisite spatial resolution, should deepen the
638 understanding of OUD.
639
640 ***Advance multiomic data integration***. The epigenomic and transcriptomic mechanisms
641 reviewed above are thought to contribute to neurophysiological changes that are ultimately
642 mediated by changes at protein level. Proteomic studies were initially not included in the
643 present systematic bibliography, considering the low number of studies available and the fact
644 that, compared to RNA- or DNA-sequencing methodologies, they are limited to the analysis of
645 a reduced genome (a few hundred proteins; see [175–178]). Nevertheless, to go further in the
646 understanding of OUD, analyses at proteomic level will be necessary, ideally in combination
647 with the characterization of aforementioned molecular layers. Unfortunately, up to now,
648 numerous epigenetic mechanisms have not yet been investigated at genome-wide scale in
649 relation to opioids, including chromatin accessibility (ATAC-Seq) and conformation (4C), or
650 histone modifications (ChIP-Seq, Cut&Tag). This contrasts with studies conducted on
651 cocaine[150, 179]. Because the latter uncovered widespread histone post-translational
652 modifications, it can be hypothesized that opioids, notably through convergent dopaminergic
653 signaling, may recruit similar types of plasticity, whose reciprocal interaction with e.g. opioid-
654 induced changes in DNA methylation or gene expression remain unknown. The next
655 challenge, therefore, will be to integrate those numerous layers of analysis into multiomic
656 models of pathophysiology, which are likely to recruit genomic locus- and cell type-specific
657 mechanisms. Illustrating these concepts, we recently conducted a human study suggesting
658 that early-life adversity and depression associate with 2 forms of DNA methylation changes in
659 the canonical CG, and the brain-enriched CAC, contexts[149]. By combining WGBS with
660 ChIP-Seq analysis of 6 histone marks, the 2 forms of plasticity were found to affect distinct
661 genomic sites and features, and to be defined by different histones modifications and
662 chromatin states. Similar multiomic investigations of opioid effects should be conducted and,
663 importantly, will require innovative bioinformatic frameworks, which are only emerging in
664 psychiatry[180]. Only 2 studies reviewed here followed that path: Mendez et al performed

665 transcriptomic and proteomic analyses, while Liu et al analyzed the transcriptome and
666 methylome (see section *4.Non-coding and epigenomic mechanisms of opioid plasticity*). In
667 both studies, multiomic integration consisted: first, in the identification of features (single
668 mRNA, DNA methylation site, or protein) passing a significance threshold, and their overlap;
669 second, in the construction of co-expression or co-methylation networks, and the prioritization
670 of groups of features, defined as modules, most significantly associated with OUD, and their
671 biological interpretation (enrichment in functional GO terms, significantly affected features,
672 GWAS results, etc). While these systems-biology approaches will undoubtedly contribute to
673 the understanding of OUD, they remain based on step-by-step aggregation of results
674 generated individually for each layer. In the coming years, it is expected that advanced
675 multiomic integration approaches (e.g. similarity network fusion, or multiblock methods[181–
676 183]), may more globally define, throughout the genome, how multiple regulatory layers
677 interact, and the chain of events linking genetic, developmental, and environmental factors to
678 abnormal behavior and compulsive drug use[184].

679 In conclusion, although numerous functional genomic studies of opioid action and OUD
680 have been conducted across animal models and patient cohorts, replicable findings appear
681 limited. In the future, combining refined behavioral models with multiomic integration, at the
682 single-cell scale, will hopefully pave the way towards the identification of innovative therapeutic
683 targets and, ultimately, contribute to better care.

684

685

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Conflict of Interest

The authors declare no conflicts of interest.

Code availability

All code used in the present review is available as Supplementary File 1.

705 **Figure legends**

706 **Figure 1. PRISMA diagram.** The review protocol was registered prospectively in Prospero
707 (record ID#CRD42022270113), and followed the Preferred Reporting Items for Systematic
708 Review and Meta-Analysis protocol (PRISMA[185]). ***Identification of studies***. Three
709 databases were used for systematic screening: MEDLINE, Embase and Web of Science. The
710 literature search was performed using the following keywords: “microarray” OR “RNA
711 sequencing” OR “bisulfite sequencing” OR “chromatin immunopurification sequencing” OR
712 “single cell RNA sequencing” AND each of the following terms: “opiates”, “opioid”, “morphine”,
713 “fentanyl”, “oxycodone”, “heroin”, “methadone” or “buprenorphine”. The initial search provided
714 2705 potentially eligible studies. ***Screening***. Articles were screened based on their title and
715 abstract, and included if they used a high-throughput, genome-wide methodology to assess
716 modifications of gene expression or epigenetic mechanisms as a function of opioid exposure,
717 in the nervous system of primates or rodents (rat, mouse), and were published in English in
718 peer-reviewed journals. Exclusion criteria included tissue other than the nervous system and
719 candidate genes approaches. Once duplicates were removed, a total of 1225 articles were
720 screened, among which 1067 were excluded because their title or abstract didn’t meet
721 eligibility criteria. ***Eligibility***. After a more thorough examination of articles’ full-text, an
722 additional 89 articles were excluded. As a result, 69 papers were selected for qualitative
723 synthesis. Among these, 44 articles reporting on transcriptomic analyses in the 5 most
724 frequently investigated regions were selected for a more detailed quantitative synthesis. Of
725 these, 37 articles investigated a single brain region, 6 covered 2 regions, and only 1
726 characterized 5 regions [13]. Overall, this resulted in 52 differential expression analyses: 18,
727 11, 11, 6 and 6 in the nucleus accumbens (NAc), frontal cortex, whole striatum, dorsal striatum
728 and spinal cord, respectively.

729

730 **Figure 2. Characteristics of studies included: Species & Sex. (A)** Species repartition in
731 studies identified during systematic search. **(B)** Distribution of sex and year of publication
732 across studies. **(C-D)** Sex distribution specifically in rodent and human studies. **(E-F)** Sample

733 sizes in rodent and human studies comparing opioid-exposed animals to controls (rodent,
734 panel E), or individuals with a diagnosis of opioid use disorder (OUD) to healthy controls.
735
736 **Figure 3. Characteristics of studies included: Injection parameters, Brain structure &**
737 **Techniques.** Among the 69 studies considered during qualitative analysis, a large panel of
738 injection parameters, nervous system structures and techniques were represented. **(A)**
739 Injection parameters used for opioid administration. NB: "mixed" refers to a single study that
740 used a combination of chronic oral administration (through bottles) and acute ip injections of
741 morphine[75]. **(B)** Number of articles investigating each nervous system structure. **(C)** Side-
742 by-side comparison of the number of articles investigating each structure with the protein
743 expression profile of the molecular target of opioids, the mu opioid receptor (MOR; as
744 assessed using [^{3}H]-DAMGO autoradiography, data in fmol/mg of tissue, adapted from[111,
745 114–116]). **(D)** Distribution of high-throughput methodologies and year of publication across
746 studies. *Abbreviations:* FCx, Frontal cortex; NAc, Nucleus accumbens; dStr, Dorsal Striatum;
747 Hc, Hippocapus; HT, Hypothalamus; Th, Thalamus; Amy, Amygdala; VTA, Ventral Tegmental
748 Area; MHb, Medial Habenula; LC, Locus Coeruleus; DRN, Dorsal Raphe Nucleus.
749
750 **Figure 4. Data availability for eligible studies and quantitative analysis in the nucleus**
751 **accumbens. (A)** The graph depicts the number of: i) differential expression analysis results
752 considered for quantitative analysis (with the corresponding number of articles between
753 brackets); ii) cases when lists of differentially expressed genes (DEG) were available; iii) cases
754 when full genome-wide results were available (with both fold changes, FC, and p-values). **(B)**
755 Number of DEG identified in studies that investigated the nucleus accumbens. **(C-E)** Venn
756 diagram of DEG identified in each species (human, mice and rat). **(F)** Venn diagram of DEG
757 in the 2 studies that investigated the dorsal striatum.
758
759 **Figure 5. Quantitative analyses in the dorsal striatum, whole striatum and frontal cortex.**
760 **(A)** Number of differentially expressed genes (DEG) identified in studies that investigated the

761 striatum (see Fig.S1 for separate analyses of studies that used acute or chronic opioid
762 administration). **(B)** Number of DEG in studies of the frontal cortex. **(C)** Number of genes
763 identified as differentially expressed (DE) across different studies. **(D)** Across the 5 brain
764 regions considered during quantitative analysis, we assessed the impact of the following 7
765 factors on consistency among studies, using pairwise Jaccard indexes: species, brain
766 structure, treatment chronicity (acute/chronic), opioid drug (morphine/heroin), strain
767 (C57BL6J/others), time of analysis following last exposure (5 lists of DEG from tissue collected
768 1 hour after opioid exposure, 5 after 2 hours, 10 after 4 hours, 4 after 8 hours, 1 after 14 hours,
769 and 3 after 24 hours) and article. For each factor (e.g., species), pair-wise JI were classified
770 in 2 categories describing whether they belonged to the same sub-group (e.g., 2 studies
771 conducted in humans; "intra"), or not (e.g., 1 study in humans compared to another in rats;
772 "inter"), within that factor. For the drug factor, studies in human were not considered as they
773 cannot be assigned to specific opioids; a single study investigated fentanyl, and was
774 excluded[33]. For the strain factor, among the 14 mouse studies available, JI significantly
775 increased among the 12 that used C57BL6J mice, while a similar analysis was not possible
776 for 129P3/J, DBA/2J, SWR/J or Kunming strains, as only 1 list of DEG was available for each.
777 One study reporting on the combined analysis of C57BL6J and DBA2J mice was excluded[54].
778 No similar analysis was possible in rats, as all available studies used Sprague-Dawley (except
779 one that did not state the strain used[67]). Note that the numbers of JI values considered in
780 each comparison are indicated at the bottom of each bar.
781
782 **Figure 6. (A-D) Threshold-free comparison of 2 bulk tissue RNA-sequencing studies**
783 **(Seney et al and Townsend et al), and cell-type specific comparison of 2 single-cell**
784 **(Avey et al) and single-nucleus (Reiner et al) RNA-sequencing studies. (A-B)** Venn
785 diagram of differentially expressed genes (DEG) identified across the 2 bulk tissue RNA-
786 Sequencing studies by Seney et al and Townsend et al (DEG defined by arbitrary significance
787 thresholds in each study: p-value < 0.05 for both) for women/female rats (A) and men/male
788 rats (B). **(C-D)** Genome-wide, threshold-free comparison of the same 2 studies using Rank-

789 Rank Hypergeometric Overlap (RRHO2) for women/female (C) rats and men/male rats (D). In
790 RRHO2, briefly, genes are ranked using p-values (signed according to directionality), from
791 upregulated (Seney: left, Townsend: bottom) to down-regulated genes (Seney: right, fentan:
792 top). Overlaps along the 2 distributions are then iteratively assessed using hypergeometric
793 testing. High p-values in the bottom-left and top-right quadrants indicates concordance,
794 whereas high p-values in the other 2 quadrants (bottom-right and top-left) indicate discordance
795 between the 2 datasets. The white crosses indicate the location of the overlap corresponding
796 to Venn diagrams depicted in panels A-B (split in subgroups of up- or down-regulated genes),
797 while black crosses correspond to most significant overlaps identified using RRHO2. **(E)**
798 Heatmap of pairwise Jaccard indexes for DEG identified across each of the 26 and 27 cell-
799 types defined by Avey et al and Reiner et al, respectively.
800

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Figure 1

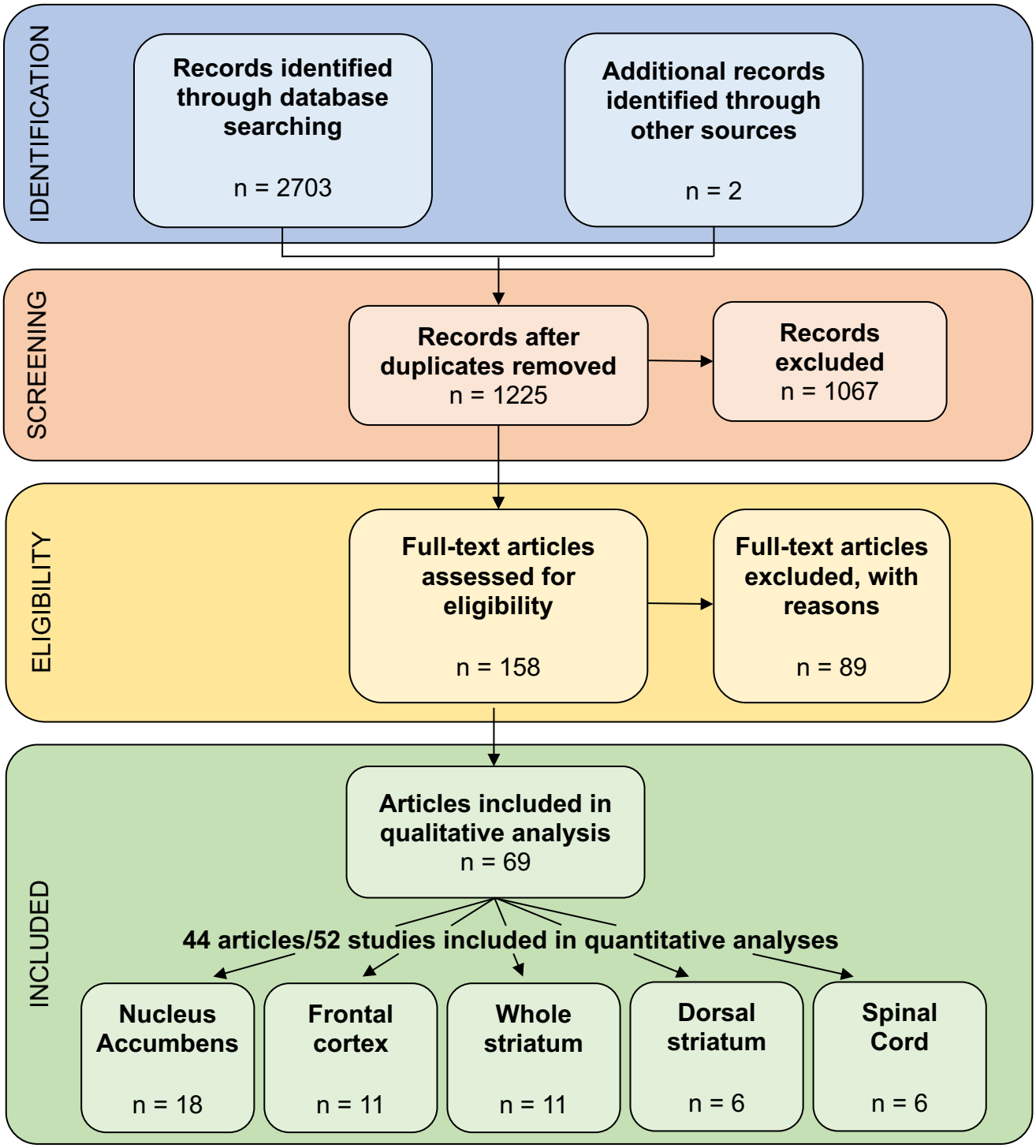

IDENTIFICATION
Records identified through database searching
n = 2703
Additional records identified through other sources
n = 2
SCREENING
Records after duplicates removed
n = 1225
Records excluded
n = 1067
ELIGIBILITY
Full-text articles assessed for eligibility
n = 158
Full-text articles excluded, with reasons
n = 89
INCLUDED
Articles included in qualitative analysis
n = 69
44 articles/52 studies included in quantitative analyses
Nucleus Accumbens
n = 18
Frontal cortex
n = 11
Whole striatum
n = 11
Dorsal striatum
n = 6
Spinal Cord
n = 6

Figure 2

A
SPECIES
Rats (n=30, 43%)
Mice (n=28, 41%)
Human (n=8, 12%)
Monkey (n=2, 3%)
Mice + rats (n=1, 1%)
B
25
20
15
10
5
0
2001-2002
2003-2004
2005-2006
2007-2008
2009-2010
2011-2012
2013-2014
2015-2016
2017-2018
2019-2020
2021
Males (n=48, 70%)
Both (n=14, 20%)
Females (n=5, 7%)
Not specified (n=2, 3%)
C
RODENT studies
Males (n=47, 80%)
Both (n=7, 12%)
Females (n=3, 5%)
Not specified (n=2, 3%)
E
HUMAN studies
Men (n=1, 12.5 %)
Men & Women
(n=7, 87.5 %)
D
Controls
Opiod treated
Vassoler, 2017
Coffey, 2020
Odegaard, 2020
Odegaard, 2021
Townsend, 2021
Borrelli, 2021
Liu, 2021
F
Controls
OUD
Albertson, 2006
Sillivan, 2013
Egervari (ATACseq), 2020
Seney, 2021
Mendez, 2021
Liu (RNAseq), 2021
Liu (bisulfite seq), 2021
Shu (bisulfite seq), 2021

Figure 3

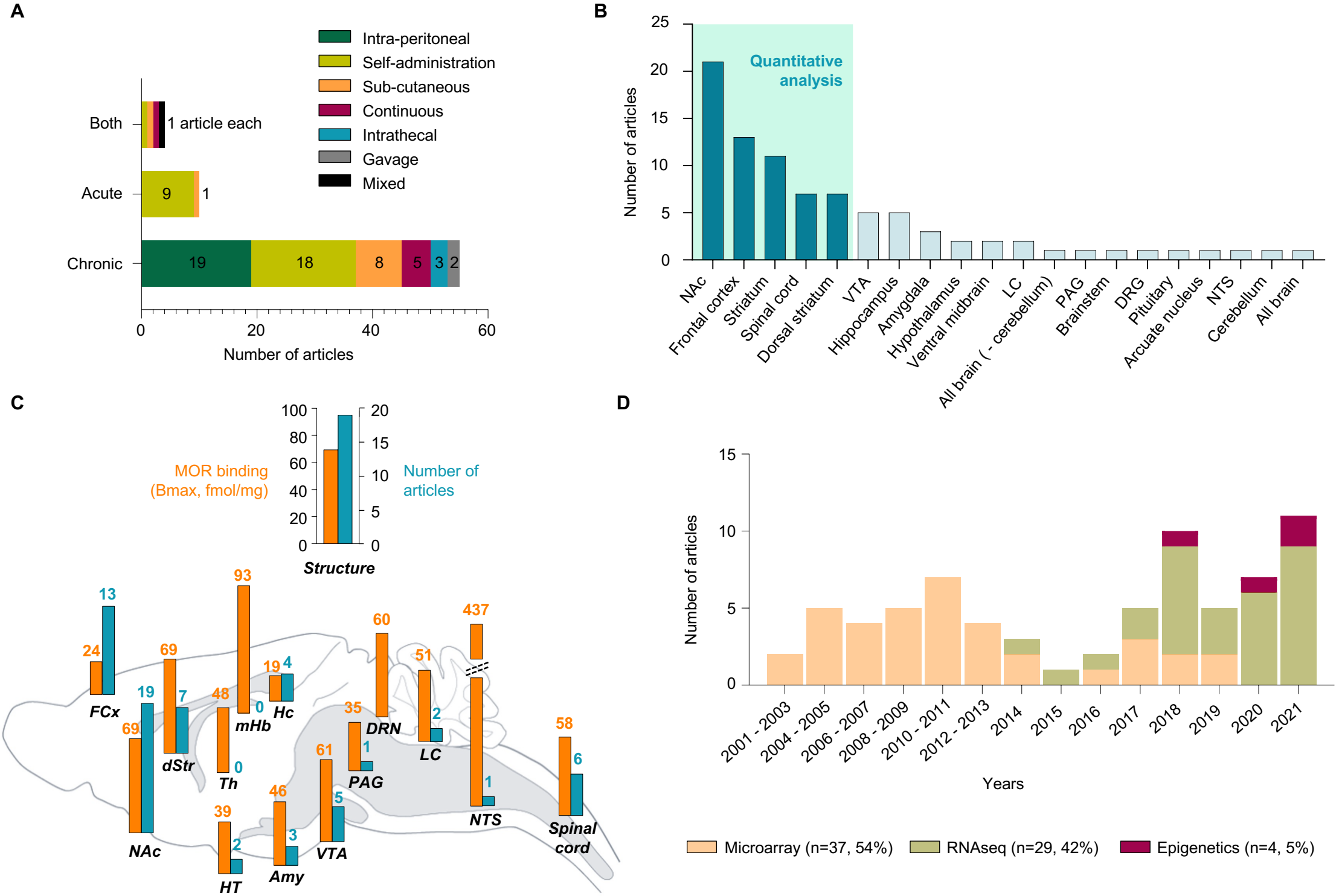

A
Intra-peritoneal
Self-administration
Sub-cutaneous
Continuous
Intrathecal
Gavage
Mixed
Both
1 article each
Acute
9
1
Chronic
19
18
8
5
3
2
0
20
40
60
Number of articles
B
Quantitative analysis
Number of articles
25
20
15
10
5
0
NAc
Frontal cortex
Striatum
Spinal cord
Dorsal striatum
VTA
Hippocampus
Amygdala
Hypothalamus
Ventral midbrain
LC
All brain (- cerebellum)
PAG
Brainstem
DRG
Pituitary
Arcuate nucleus
NTS
Cerebellum
All brain
C
MOR binding (Bmax, fmol/mg)
Number of articles
Structure
FCx
24
13
NAc
69
19
dStr
69
7
HT
39
2
Th
48
0
mHb
93
0
Hc
19
4
Amy
46
3
VTA
61
5
DRN
60
PAG
35
1
LC
51
2
NTS
437
1
Spinal cord
58
6
D
Number of articles
Years
2001 - 2003
2004 - 2005
2006 - 2007
2008 - 2009
2010 - 2011
2012 - 2013
2014
2015
2016
2017
2018
2019
2020
2021
Microarray (n=37, 54%)
RNAseq (n=29, 42%)
Epigenetics (n=4, 5%)

Figure 4

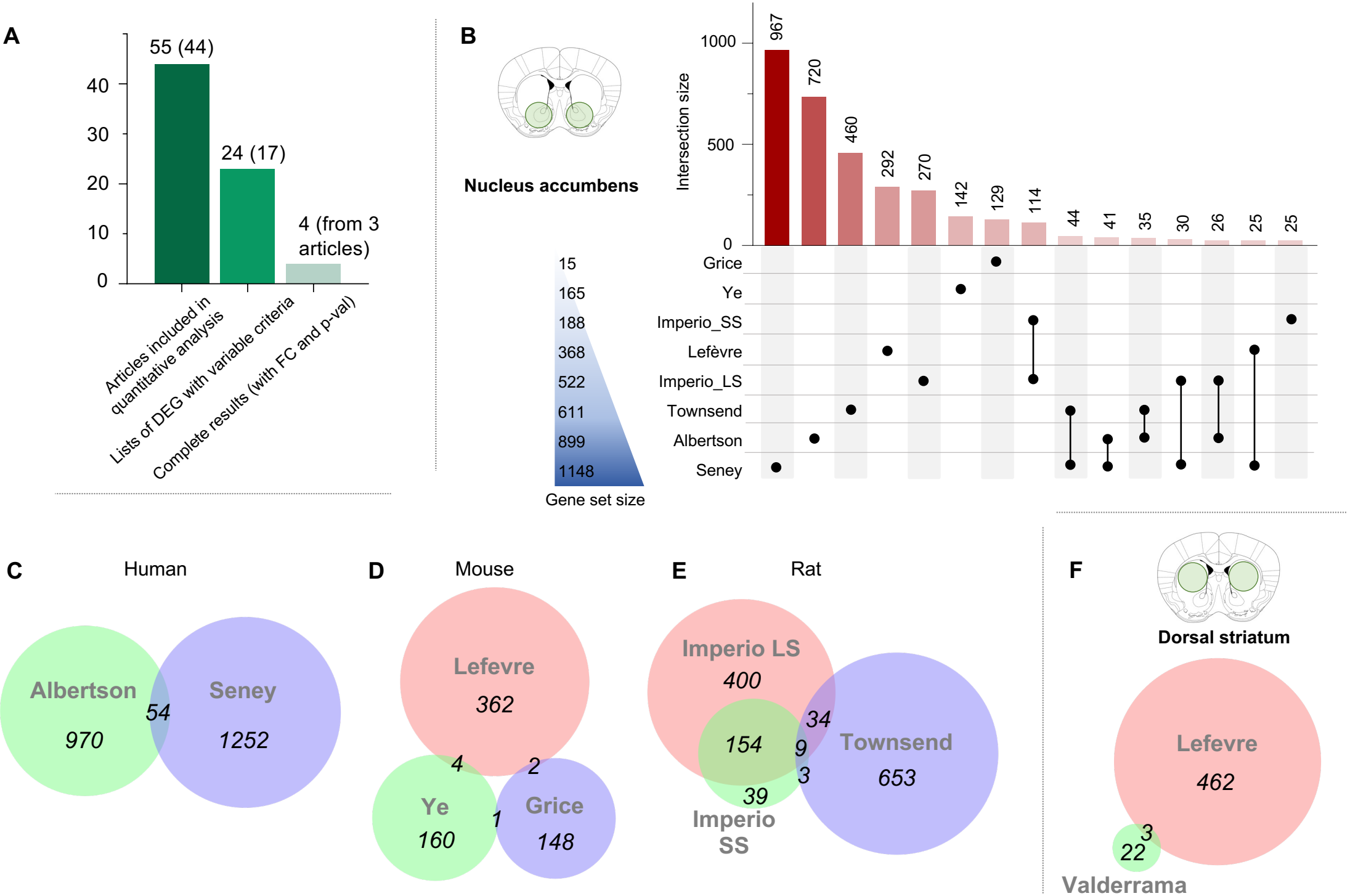

A
55 (44)
24 (17)
4 (from 3 articles)
0
10
20
30
40
Articles included in quantitative analysis
Lists of DEG with variable criteria
Complete results (with FC and p-val)
B
Nucleus accumbens
Intersection size
0
500
1000
967
720
460
292
270
142
129
114
44
41
35
30
26
25
25
Grice
Ye
Imperio_SS
Lefèvre
Imperio_LS
Townsend
Albertson
Seney
15
165
188
368
522
611
899
1148
Gene set size
C
Human
Albertson
970
54
Seney
1252
D
Mouse
Lefevre
362
4
2
Ye
160
1
Grice
148
E
Rat
Imperio LS
400
154
34
9
3
39
Imperio SS
Townsend
653
F
Dorsal striatum
Lefevre
462
3
22
Valderrama

Figure 5

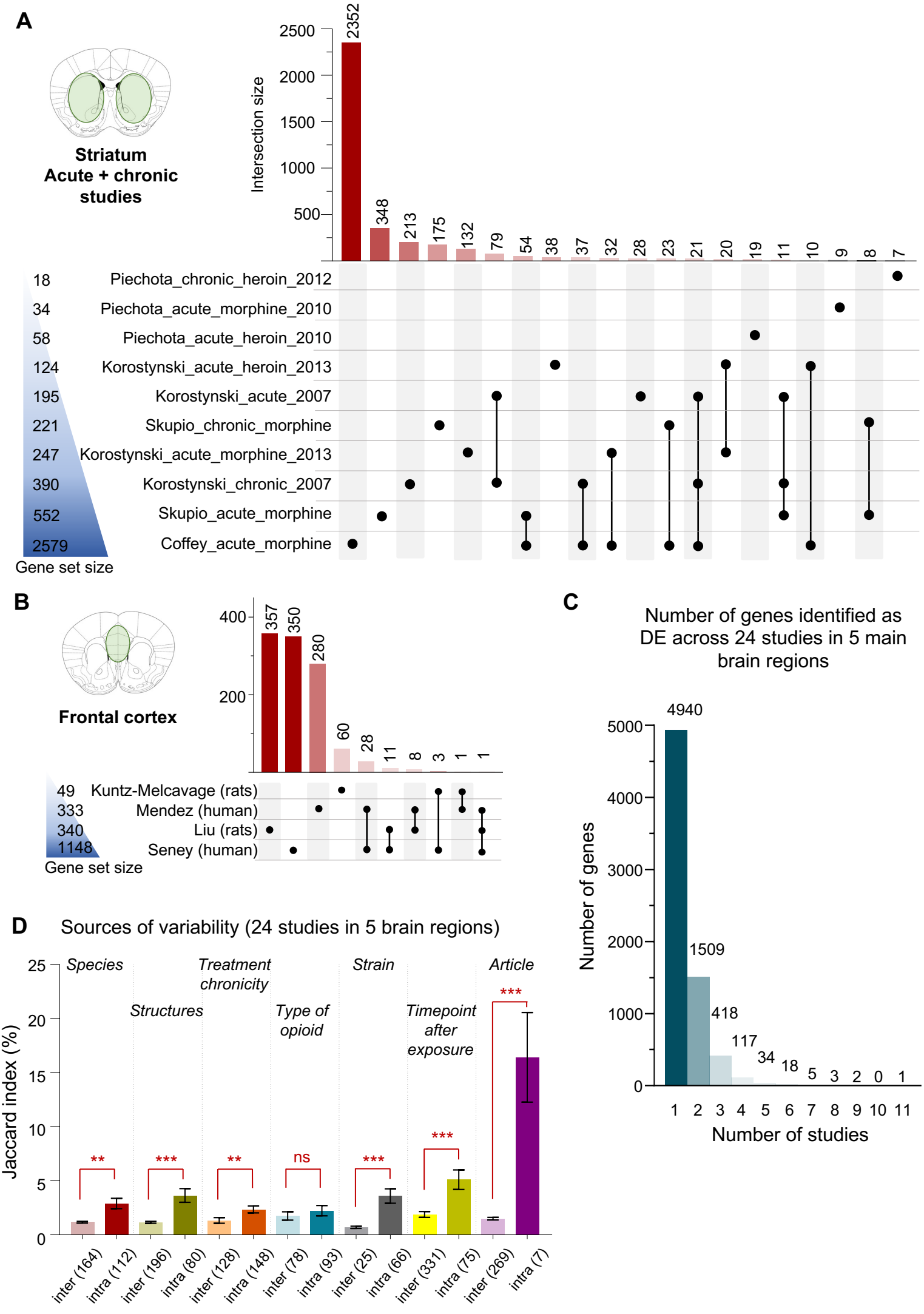
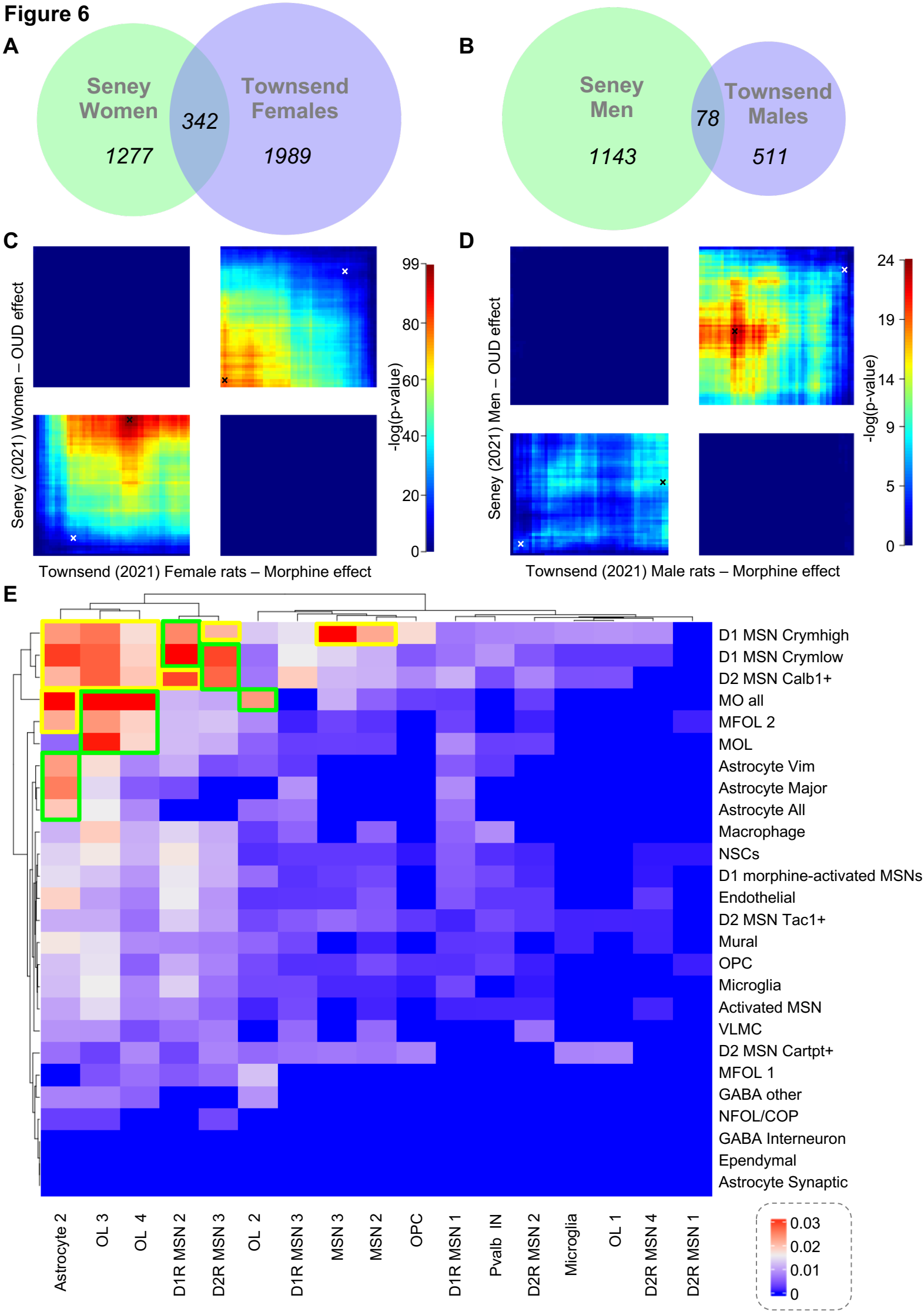

A
Striatum
Acute + chronic studies
Intersection size
2352
348
213
175
132
79
54
38
37
32
28
23
21
20
19
11
10
9
8
7
18 Piechota_chronic_heroin_2012
34 Piechota_acute_morphine_2010
58 Piechota_acute_heroin_2010
124 Korostynski_acute_heroin_2013
195 Korostynski_acute_2007
221 Skupio_chronic_morphine
247 Korostynski_acute_morphine_2013
390 Korostynski_chronic_2007
552 Skupio_acute_morphine
2579 Coffey_acute_morphine
Gene set size
B
Frontal cortex
357
350
280
60
28
11
8
3
1
1
49 Kuntz-Melcavage (rats)
333 Mendez (human)
340 Liu (rats)
1148 Seney (human)
Gene set size
C
Number of genes identified as DE across 24 studies in 5 main brain regions
Number of genes
4940
1509
418
117
34
18
5
3
2
0
1
Number of studies
D
Sources of variability (24 studies in 5 brain regions)
Jaccard index (%)
Species
Structures
Treatment chronicity
Type of opioid
Strain
Timepoint after exposure
Article
**
***
**
ns
***
***
***
inter (164)
intra (112)
inter (196)
intra (80)
inter (128)
intra (148)
inter (78)
intra (93)
inter (25)
intra (66)
inter (331)
intra (75)
inter (269)
intra (7)

Figure 6


A
Seney Women
1277
342
Townsend Females
1989
B
Seney Men
1143
78
Townsend Males
511
C
Seney (2021) Women – OUD effect
Townsend (2021) Female rats – Morphine effect
-log(p-value)
99
80
60
40
20
0
D
Seney (2021) Men – OUD effect
Townsend (2021) Male rats – Morphine effect
-log(p-value)
24
19
14
9
5
0
E
D1 MSN Crymhigh
D1 MSN Crymlow
D2 MSN Calb1+
MO all
MFOL 2
MOL
Astrocyte Vim
Astrocyte Major
Astrocyte All
Macrophage
NSCs
D1 morphine-activated MSNs
Endothelial
D2 MSN Tac1+
Mural
OPC
Microglia
Activated MSN
VLMC
D2 MSN Cartpt+
MFOL 1
GABA other
NFOL/COP
GABA Interneuron
Ependymal
Astrocyte Synaptic
Astrocyte 2
OL 3
OL 4
D1R MSN 2
D2R MSN 3
OL 2
D1R MSN 3
MSN 3
MSN 2
OPC
D1R MSN 1
Pvalb IN
D2R MSN 2
Microglia
OL 1
D2R MSN 4
D2R MSN 1
0.03
0.02
0.01
0