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 2709 articles identified, 73 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 interacting biological, psychological and socio-economic factors[4]. At a 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 contribution of candidate gene studies (already reviewed in the past[6, 7]), this focus on genome-wide analyses reflects the

conviction that understanding heterogeneous phenotypes such as OUD requires 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, 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, 73 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=73 studies)

103 ***Species and sex ratio.*** Most studies were conducted in rodents (Fig.2A): 30 (41%) in rats,
104 29 (40%) in mice, and 1 in both species. Only a small number investigated humans (n=11,
105 15%) and macaques (n=2, 3%), reflecting well-known practical limitations. As consequence,
106 current knowledge on species-specific opioid effects is limited.

107 Regarding sex, epidemiological surveys indicate that OUD prevalence is higher in
108 males than in females[3, 14] (although smaller differences have been reported with DSM-5
109 criteria[15]), with a number of sex-specific risk factors (e.g., ethnicity, income). This clinical
110 situation has long been modeled in rodents, as sex differences in behavioral effects of
111 opioids[16] have been described for analgesia[17], tolerance[18], withdrawal symptoms[19],
112 reward[20] or motivation[21]. In comparison, molecular mechanisms underlying these
113 differences are poorly characterized. The majority of OUD studies reviewed here investigated
114 males only (n=51, 70%; Fig.2B-D), with few conducted in females only (n=5, 7%), and 15
115 (20%) that included both sexes (7 in rodents, 8 in humans). These numbers are consistent
116 with the current landscape of neuroscience research, in which the ratio of articles reporting on
117 males only, as opposed to females only, is around 5 to 1[22]. We nevertheless note an
118 encouraging trend, as the majority of studies published since 2021 (8/12) investigated both
119 sexes. Several factors likely underlie this evolution: policies from funding agencies (such as
120 the NIH; https://grants.nih.gov/grants/guide/notice-files/not-od-15-102.html); increased
121 recognition that females do not display more experimental variability than males[23]; and
122 evidence that pathophysiological mechanisms may differ across sexes, as shown for
123 depression[24], schizophrenia[25] or alcohol use disorder[26].

124 Specifically in rodents (Fig.2C), 80% of studies investigated males only (n=48). Seven
125 articles investigated both sexes (n=7, 12%), using surprisingly low sample sizes (n=1-4 in
126 male/female, and opioid-treated/control, groups; Fig.2D). Among these, 2 studies treated sex
127 as a covariate[27, 28], and 3 pooled both sexes for the analysis of opioid effects[29–31],
128 therefore providing no direct description of male/female differences. This is concerning,
129 considering that the 2 remaining studies found little overlap, from 5 to 35% (see[32] and[33]),
130 among morphine-induced changes occurring in each sex. Therefore, available evidence

131 points toward substantial sex-specificity in opioid-induced transcriptomic plasticity, which will
132 have to be confirmed in future work. In human, even less is known (Fig.2E). Among 11 articles,
133 8 investigated males and females, while 3 investigated males only. However, these 8 studies
134 showed a strong bias towards examination of men (Fig.2F), with only one using similar sample
135 sizes in both sexes[34]. In the latter, sex was treated as a covariate, with no specific
136 description of its impact. Altogether, this significant gap in the literature calls for additional
137 work, with the hope that better understanding of sex-specific pathophysiological routes may
138 unravel distinct and more efficient therapeutic strategies.
139
140 ***Route and duration of opioid administration.*** Among studies included in qualitative analysis
141 (n=73), the majority used chronic administration (n=59/73, 81%; see Fig.3A), mostly via self-
142 administration (SA, n=22/59, 37%, including 11 human studies) and intraperitoneal injection
143 (ip; n=19, 32%), followed by subcutaneous injection (n=8, 14%). Other procedures were less
144 frequently used: continuous administration (with pellets or mini-pumps; n=5, 8%), intrathecal
145 administration (n=3, 5%), or gavage (n=2, 3%).
146 There is limited information regarding differences among administration procedures.
147 Interestingly, Lefevre et al[35] recently compared 3 conditions: continuous and systemic
148 morphine infusion using osmotic mini-pumps, with ("Interrupted") or without ("Continuous") the
149 induction, twice daily, of a precipitated withdrawal episode (using naloxone, a non-specific
150 opioid antagonist), compared to Controls (saline mini-pumps). Results indicated 687 and 407
151 differentially expressed genes (DEG) when comparing Interrupted and Control groups in the
152 NAc and dorsal striatum, respectively, with only 1 DEG between Continuous and Control
153 groups. Therefore, rather than opioid exposure per se, it is the repetition of intermittent
154 withdrawal episodes that may drive the intensity of transcriptomic adaptations affecting the
155 mesolimbic system (whether precipitated, as in this study, or spontaneous, in-between bolus
156 injections). This is consistent with the clinical notion that the alternation of intoxication and
157 withdrawal phases is critical for the development of OUD.

158 Opioid effects have also been investigated following acute administration (10/73
159 studies, 14%), corresponding mostly to ip injection (9 articles, with only 1 using subcutaneous
160 injection). While these studies were interested in the use of opioids as analgesics or
161 anesthetics[36, 37], or compared acute effects of various psychoactive drugs[38], their results
162 may be relevant for the understanding of OUD. Indeed, comparisons of inbred mouse strains
163 found striking variability in the intensity of opioid physical dependence, with the same strains
164 showing severe or mild physical signs when withdrawal was precipitated after acute or chronic
165 opioid injections[39, 40]. In other words, there may be shared genetic determinants, within
166 each strain, for brain adaptations triggered by acute or chronic opioid exposure. A recent
167 single-cell RNA-sequencing study (scRNA-Seq) provides an interesting illustration of how
168 such strain-specific genetic traits may translate into at least partially similar transcriptional
169 plasticity during acute or chronic morphine exposure ([41] and *3. High cellular resolution*
170 *approaches* below).

171 Another important consideration is that most studies (51/73, 70%) used passive
172 administration, which do not model volitional or compulsive drug-taking. This reflects the
173 technical difficulty of intravenous SA experiments (IVSA), particularly in the mouse. Few
174 studies directly compared passive and active exposure. Among these, Tapocik et al[42]
175 analyzed “yoker” mice and groups of “yoked-morphine” or “yoked-saline” animals which, each
176 time the yoker self-administered morphine, were passively exposed to morphine or saline IV
177 injections. In C57BL/6J, much more DEG were identified between the yoker and yoked-saline
178 groups (n=1176) than between yoked-morphine and yoked-saline (n=244), with their overlap
179 likely reflecting passive morphine effects (n=103). In DBA/2J mice, which did not develop
180 morphine self-administration (as shown by the absence of differences in operant responding
181 among the 3 groups), transcriptional effects were milder, as expected (yoked-morphine *vs*
182 yoked-saline, n=262 DEG; yoker *vs* yoked-saline, n=107). This suggests that distinct
183 transcriptional regulations may mediate voluntary opioid consumption and reflect their
184 pharmacological effects, with both aspects ultimately contributing to OUD.

185 New paradigms have been developed to facilitate such studies, which notably include
186 oral SA[43–45]), or devices for opioid vapor SA[46]. In addition, optogenetic tools now enable
187 light-induced self-stimulation (optogenetic intracranial self-stimulation, oICSS)[47]. The latter
188 models are based on selective manipulation, using opsins, of the activity of anatomically- or
189 genetically-defined neuronal populations. While these approaches do not recapitulate the
190 systemic effects of opioid IVSA, they are nevertheless able to trigger similar behavioral
191 deficits, including uncontrolled consumption, resistance to punishment, or excessive
192 motivation[48, 49]. It is therefore possible that they may allow dissecting the neuronal
193 pathways that are necessary or sufficient for each of these behavioral dimensions, as well as
194 underlying molecular mechanisms. Another avenue for improvement relates to the mode of
195 action of genetic tools used for oICSS. Upon light-stimulation, opsins open ion channels that
196 modulate neuronal excitability. Their high temporal resolution does not necessarily represent
197 the best model for the longer time-frame of pharmacological action of drugs of abuse. To more
198 faithfully mimic such kinetics and associated intracellular signaling, chemogenetics or chimeric
199 opsins (for light-induced metabotropic signaling, with a proof-of-concept available for
200 MOR[50]) might represent better tools.
201
202 ***Nervous system structures.*** Among articles eligible for qualitative analysis, 18 regions of the
203 nervous system were explored (Fig.2B). Unsurprisingly, a large majority focused on the
204 mesocorticolimbic dopaminergic pathway: the NAc (22 articles[13, 27, 30, 33–35, 37, 51–65]),
205 frontal cortex (16; [13, 29, 32, 34, 62, 66–76]), whole/unspecified striatal complex (11; [31, 38,
206 77–85]), dorsal striatum (7; [13, 35, 59–61, 86, 87]) and ventral tegmental area (VTA, 5; [33,
207 62, 88–90]). Other regions included the spinal cord (7; [77, 91–96]); hippocampus (5; [13, 78,
208 97–99]); amygdala (3; [13, 100, 101]); locus coeruleus[89, 102], ventral midbrain[42, 103],
209 hypothalamus[104, 105] (2 each), whole brain with[106] or without[107] cerebellum,
210 periaqueductal gray matter[108], pituitary gland[105], arcuate nucleus[109], nucleus of the
211 tractus solitarius[36], brainstem[28], cerebellum[99] or dorsal root ganglia[110] (DRG, 1 each).
212 Below, we briefly describe the rationale for studying such diverse structures.

The course of OUD involves 3 stages: intoxication, withdrawal/negative affect, and anticipation of next intoxication. These stages reflect gradual adaptation of the brain to drug exposure, and rely on distinct mechanisms. The intoxication stage, mostly driven by acute reward, notably associates with dopamine release, in the striatum and frontal cortex, by neurons located in the VTA. The second stage corresponds to negative reinforcement, whereby drug consumption alleviates physical and negative affective states of withdrawal, involving the locus coeruleus[111], NAc[112], and amygdala[113], among others. Finally, anticipation results from memories of drug-associated cues and contexts (hippocampus), as well as impaired goal-directed behaviors, implicating higher-order structures (e.g. prefrontal and orbital cortices[114]). These relationships are not exclusive, with some structures implicated at multiple stages (e.g., the striatum, also involved in habit formation). Other regions (spinal cord, DRG, brainstem, periaqueductal gray matter, PAG) were investigated in relation to opioid analgesia, tolerance and hyperalgesia. Finally, few articles were interested in how opioids modulate neuroendocrine systems (hypothalamus, arcuate nucleus, pituitary gland).

In addition to these associations, it is also important to consider the distribution of the endogenous opioid system. This system is composed of 3 opioid receptors (mu, delta and kappa; MOR, DOR and KOR), among which the MOR is necessary for opioid-induced analgesia, reward, and physical dependence[115]. Neurons expressing this receptor therefore represent pharmacological entry points for brain adaptations to opioids. Comparison of studies reviewed here with MOR brain distribution (assessed using ligand autoradiography[116–121]; Fig.3C) indicates that: i) MOR is highly expressed among structures most frequently studied, as expected; ii) surprisingly, other areas with similarly high expression have received little to no attention (NTS, locus coeruleus, dorsal raphe nucleus, medial habenula, thalamus), calling for more work.

Techniques. Thirty-seven and 29 studies used microarrays and RNA-seq, respectively, to analyze the transcriptome (Fig.3D). While the first RNA-seq study was published in 2014[37], this technique has since progressively replaced microarrays, reflecting well-recognized

241 advantages (higher sensitivity and throughput, gene discovery). For epigenomic profiling, 3
242 studies used DNA methylation arrays[73, 75, 76], 2 bisulfite sequencing[72, 74], and 1
243 ATACseq (Assay for Transposase-Accessible Chromatin with sequencing)[87].
244

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

246 To identify reproducible transcriptomic opioid signatures, we ran quantitative analyses of bulk-
247 tissue results available for the 5 most studied structures (44 articles; Fig.4A). To do so, gene-
248 level results were retrieved when available. This yielded 24 lists of DEG comparing opioid-
249 treated animals and control groups, originating from 17 articles. Among these, 6 articles did
250 not report any p-values, while the remaining 11 used very diverse thresholds on p-values and
251 fold changes (FC; Supplementary Table 1). Only a small minority (3/44, 6.8%[31, 33, 34])
252 provided p-values and FC for all genes. In addition, raw data were available in the public
253 repository GEO for less than half of the studies that reported DEG (8/17, 47%). Overall, this
254 underscores strong heterogeneity in data analysis and reporting, which limits re-use[122]. To
255 counter such issues, efforts are being made to harmonize practices[123, 124], with guidelines
256 enforced by funding agencies[125]. For the present review, a genome-wide meta-analysis
257 combining effect and sample sizes across studies was not possible. We nevertheless: i)
258 Systematically assessed concordance among available lists of DEG; ii) Conducted a
259 threshold-free comparison of the 2 NAc studies for which genome-wide results were available.
260

261 **2.1 Nucleus accumbens**

262 Among 18 articles, only 8 provided lists of DEGs: 4 in mice (males only[35, 37, 51, 54]), 2 in
263 rats (1 in males[56], 1 in both sexes[33]), 2 in humans (both sexes[34, 58]). The 8 articles
264 provided a total of 3502 DEG (Fig.4B-E)**.** Among these, the vast majority were identified by 1
265 study only (n=3017/3502, 86%), while 424, 57 and 4 DEG were identified across 2, 3 or 4
266 studies, respectively. No single genes were common to 5 or more articles. In addition, the best
267 overlap was found among the 2 DEG lists reported in a single study by Imperio et al[56]
268 (Fig.3E). Overall, this indicates low concordance among NAc studies.

269 To define factors contributing to this heterogeneity, we intersected DEG lists in each
270 species separately (Fig.3C-E). This, however, did not improve concordance: in humans, only
271 54 genes were common to both studies (Jaccard Index, JI=2.4%, Fig.4C, Supplementary
272 Table 2 for the matrix of pair-wise JI across all studies); in mice, 7 were identified by at least
273 2 studies (JI=1%, Fig. 4D); in rats, the JI increased to 15.5% (Fig.4E), mostly driven by high
274 overlap among the 2 aforementioned DEG lists from Imperio et al. In the latter work, the
275 authors identified large- (LS) and small-suppressors (SS): in LS rats, greater avoidance of a
276 natural reward (saccharine) that predicted heroin availability was associated, compared to SS
277 rats, with worse outcomes during later heroin IVSA (increased consumption, higher motivation
278 and relapse). Gene expression changes occurring in the LS group (compared to control rats
279 given access to saccharine but not heroin; n=597 genes), and in the SS group (same controls,
280 n=205) showed a JI of 25.5% (with striking concordance in up- and down-regulation). This
281 was much higher than overlap between Imperio et al and the other rat study, by Townsend et
282 al (Imperio-SS/Townsend, JI=1.35%; Imperio-LS/Townsend, 3.43%), suggesting that
283 differences across laboratories and animal facilities may significantly contribute to poor
284 concordance. Importantly, while the relatively small number of studies in the NAc precluded
285 definitive conclusions, a more powerful analysis of sources of variability was possible across
286 our 5 regions of interest (see section *2.6*).
287
288 **2.2 Dorsal striatum**
289 Only 2 dorsal striatum studies provided DEG (Fig.3F). One study used chronic morphine
290 administration interrupted by naloxone injections in male mice[35], as described above, while
291 the other used acute morphine injection in male rats[86]. Reflecting these differences, a very
292 small overlap was found between the 2 (0.62%). Therefore, while the dorsal striatum is thought
293 to gradually gain control over drug-taking in OUD[126], underlying transcriptomic plasticity is
294 poorly characterized.
295
296 **2.3 Whole striatum**

297 Six of the 11 whole striatum studies provided DEG[31, 38, 79–82] (Fig.5B): 2 investigated
298 chronic heroin[82] or morphine[31], while 2 compared acute effects of distinct opioids
299 (morphine and heroin)[38, 80], and 2 compared acute and chronic morphine[79, 81]. Most
300 DEG were identified by 1 study only (3308/3736, 88.5%), with only 112 genes common to 3
301 or more studies. Consistent with NAc results, strongest overlaps were observed for lists of
302 DEG coming from the same article, with for example 54 (JI=17%, Korostynski 2013[38]) and
303 16 (21%, Piechota 2010[80]) DEG common to acute morphine and heroin injections,
304 respectively, and 139 DEG (31%, Korostynski 2007 [81]; Fig.5A) common to acute and chronic
305 morphine administration. In sharp contrast, comparisons across, rather than within, studies
306 yielded poor overlaps, even when considering those that used similar drug administration:
307 among chronic morphine studies (Fig.S1A), the JI varied from 0.1 to 2.6% (Korostynski
308 2007/Skupio 2017), and, among acute morphine studies, from 2.7% to 7.8% (Fig.S1B), with
309 only one comparison standing out among acute heroin studies (30 common DEG, JI=19%,
310 Piechota 2010/Korostinsksy 2013). Importantly, 5 of these 6 studies in the whole striatum were
311 conducted by the same group, suggesting that significant variability may persist even among
312 studies conducted in the same facility, but at different periods (see also section 2.6).
313
314 **2.4 Frontal cortex**
315 Seven studies provided DEG (Fig.5B): 2 in humans[34, 70], 5 in rats[29, 32, 67–69]. Data from
316 Odegaard et al[29] were discarded, as they corresponded to transgenerational effects in
317 animals not directly exposed to opioids. For 2 additional studies [67, 69], we could not map
318 microarray probes to genes. Among the 4 remaining articles, little overlaps were again found
319 (Kuntz-Melcavage/Mendez, JI=0.5%; Kuntz-Melcavage/Seney, 1.1%; Liu/Mendez, 1.4%;
320 Liu/Seney, 1.6%), with no improvement within species (Liu/Kuntz-Melcavage in rats, JI=0%;
321 Seney/Mendez in humans, 4.1%).
322
323 **2.5 Spinal cord**

324 Among the 6 spinal cord studies, 5 did not provide DEG[91–95], while the 6th one used a
325 homemade microarray[77], for which correspondence between probe IDs and genes was only
326 partially provided. As such, no comparison was possible.
327
328 **2.6 Sources of variability**
329 Considering that pharmacological recruitment and intra-cellular signaling of MOR may trigger
330 a set of common transcriptional adaptations across the 5 regions of interest, we next pooled
331 all lists of DEGs (Fig.5D). Again, the vast majority of DEG (70.1%, 4940/7047) were unique to
332 a single study, while only 21.4% common to 2 studies (1509), 5.9% to 3 (418), and 1.7% to 4
333 or more (117; Supplementary Table 3). Interestingly, Cdkn1a (Cyclin Dependent Kinase
334 Inhibitor 1A) was identified by 11 studies. This gene encodes for p21, a protein involved in the
335 regulation of the cell-cycle and oligodendrocytes[127]. Avey et al[51] found Cdkn1a to be one
336 of the most significantly upregulated genes in response to acute morphine. This upregulation
337 occurred in oligodendrocytes, and was blocked by the opioid antagonist naltrexone, indicating
338 a MOR-dependent mechanism. Of note, cocaine, another major drug of abuse, similarly
339 recruits p21, as cocaine-induced behavioral responses were modified in p21 knockout
340 mice[128]. Using this knock-out line, or viral approaches for p21 manipulation, represent
341 appealing perspectives to further investigate this gene in OUD.
342 We also performed Gene Ontology (GO) analysis using DEG common to at least 3, 4
343 or 5 studies (Supplementary Table 4). No results were significant for the small list of 63 DEG
344 common to 5 or more studies. When considering the 180 DEGs common to at least 4 studies,
345 only 3 GO terms were enriched: cell periphery, plasma membrane and hormone activity.
346 Results more directly related to the nervous system emerged for the 598 DEG common to at
347 least 3 studies, with 46 significant terms partly related to synaptic signaling and cell-cell
348 communication, which may reflect general opioid-induced electrophysiological and synaptic
349 adaptations across brain structures.
350 Because weak DEG overlaps were identified within individual regions (even when
351 grouping studies based on species, Fig.4C-E, or treatment chronicity, Fig.S1), we sought to

352 more systematically assess, across the 5 regions, the impact of following factors: species,
353 brain structure, treatment chronicity, drug, strain, time-point of assessment following last
354 exposure, and article (Fig.5D). For each factor (e.g., species), pair-wise JI were classified in
355 2 categories depending on whether they belonged to the same sub-group within that factor (2
356 studies in humans; “intra”), or not (1 human study compared to another in rats; “inter”). As
357 expected, grouping list of DEG by species (p=2.5E-3), brain structures (3.4E-8), treatment
358 chronicity (p=1.4E-3), mouse genetic background (3.9E-5) or the time-point of analysis after
359 opioid exposure (6.7E-10) all increased concordance, with higher JI. This is consistent with
360 the notion that, although they remain poorly characterized, these 5 variables contribute to
361 opioid-induced transcriptional plasticity. In contrast, the type of opioid used had no significant
362 impact (p=0.059) when considering the 13 and 5 rodent DEG lists related to morphine or
363 heroin, respectively (human studies were discarded, as the types of opioids consumed are
364 diverse and poorly characterized). This is surprising, considering that these 2 MOR agonists
365 exhibit strong pharmacokinetic differences and would be expected to generate distinct
366 adaptations (heroin being a lipophilic prodrug that rapidly crosses the blood brain barrier to be
367 metabolized in 6-monoacetylmorphine and morphine, which in turn activate MOR). Time-
368 course and dose-response experiments may be necessary to detect such differences.
369 Importantly, concordance increased most when DEG lists came from the same publication
370 (p=2.5E-5), despite the fact that only 7 comparisons fell under this category. As already
371 mentioned, this suggests that differences across laboratories may explain a substantial part
372 of the lack of concordance across studies.

373 Finally, we refined our analysis by considering the directionality (up/down) of DEG,
374 focusing on genes identified by at least 5 studies (n=63). Among results from 17 out of 24
375 studies that reported on directionality (Fig.S2), most genes (37/63, 59%) showed discordant
376 results (while, for 8 others, directionality was reported by only 1 or no study). Only 18 genes
377 showed 100% concordance across studies (29%; 17 up-regulated, 1 down), among which 11
378 were identified by at least 3 studies. The latter included the aforementioned Cdkn1a, as well
379 as Slc2a1, Fkbp5, Sult1a1, Arrdc3, Ccdc117, Plin4, Wscd1, Arid5b, Pla2g3, and Tsc22d3.

380 Slc2a1 encodes for the glutamate transporter GLT1, which plays an important role in
381 extracellular glutamate uptake and the regulation of excitatory transmission. A recent study
382 showed that during prolonged morphine withdrawal, activation of KOR in the amygdala leads
383 to increased GLT1 expression and excitatory drive in the NAc, thereby mediating withdrawal-
384 induced depressive-like behaviors. Another interesting hit is Fkbp5, a chaperone and intra-
385 cellular negative regulator of glucocorticoid signaling that has been largely investigated in
386 stress-related disorders[129], including OUD[130]. The 8 remaining genes, although
387 comparatively less studied in relation to opioids (Fig.S2), represent candidates for future work.
388

389 **2.7 Threshold-free genome-wide comparison**

390 Next, we compared the 2 NAc studies for which full genome-wide results were available
391 (Seney et al[34], Townsend et al[33]). While both included males and females, only Townsend
392 reported sex-specific results. Therefore, we reprocessed Seney's raw data to identify DEG in
393 each sex separately (*Supplementary Material*). This slightly increased concordance, arguing
394 for the importance of accounting for sex: while JI were equal to 7.5 and 4% when comparing
395 initial Seney data (male/females pooled) with Townsend data in females or males, respectively
396 (Fig.S3A-B), they increased to 9.5% and 4.5% when considering Seney's sex-specific results
397 (Fig.6A-B).

398 We then used RRHO2 (Rank–Rank Hypergeometric Overlap[131]) to compare the 2
399 studies. RRHO2 iteratively performs hypergeometric testing for all combinations of thresholds
400 applied to each dataset, generating a "threshold-free" analysis. Because this approach takes
401 directionality into account, we also computed, for comparison, overlaps among either up- or
402 down-regulated DEG reported by each study (white crosses, Fig.6C-D). Compared to the
403 latter, much more significant overlaps were identified when RRHO2 considered less stringent
404 p-value thresholds (best hypergeometric results are shown as black crosses). By definition,
405 these increased overlaps corresponded to large gene lists which, although commonly
406 dysregulated in similar direction, individually exhibited milder FC. Interestingly, these overlaps
407 were detected despite the fact that Seney investigated human post mortem tissue from

408 individuals with OUD, while Townsend focused on rat tissue collected after 24 hours of
409 withdrawal from fentanyl IVSA. Also, different patterns of convergence were observed in each
410 sex: in females, overlaps were highly significant among both up- and down-regulated genes
411 while, in males, a lower concordance predominantly affected down-regulated genes. This
412 suggests that the rat dataset may differentially capture sex-specific signatures of the human
413 disorder. Overall, these analyses illustrate the utility of reporting full genome-wide data which,
414 in combination with threshold-free approaches, may generate deeper understanding (see
415 *Discussion*).
416
417 **3. High cellular resolution approaches**
418 Studies reviewed above indicate that opioids induce genome-wide transcriptomic changes,
419 with striking variability across laboratories, experimental designs, and brain regions. This likely
420 reflects a combination of: i) direct pharmacological opioid effects, which correspond to the
421 recruitment of MOR intracellular signaling and result in decreased neuronal excitability[132];
422 ii) indirect effects, as the chemical identity and connectivity pattern of MOR-expressing cells
423 determine how their inhibition leads to secondary changes affecting other
424 neurotransmitters[117]. This raises 2 questions: how is each cell type, or even each brain cell,
425 affected by these changes? What are underlying molecular regulatory principles?
426 To address the first question, several groups recently used cell-type specific and
427 single-cell methodologies. Coffey et al[31] focused on microglial cells of the whole striatum,
428 using translational ribosome affinity purification (TRAP, to capture ribosomes and the
429 "translating transcriptome"[133, 134]). Chronic morphine effects were analyzed with, or
430 without, the induction of naloxone-precipitated withdrawal. Results showed that both chronic
431 morphine and withdrawal triggered numerous changes in gene expression, which mostly
432 affected GO terms related to cAMP signaling, regulation of synapses, and the cytoskeleton. A
433 striking negative correlation was observed between the 2 sets of results, providing a correlate,
434 at the level of the microglial transcriptome, of the pharmacological antagonism between
435 morphine and naloxone. Considering the inter-strain variability in the sensitivity to both acute

436 and chronic opioid physical dependence described earlier (see section *1.Qualitative analysis-*
437 *Route and duration of opioid administration* and[39, 40]), an interesting perspective would be
438 to determine whether naloxone similarly antagonizes transcriptomic effects of acute opioid
439 exposure, and how this is modulated by underlying genetic variation. Of note, surprisingly,
440 such cell-type specific approaches have not been used to interrogate opioid effects in neuronal
441 cells, let alone opioid-responsive MOR-expressing neurons which, arguably, may be
442 particularly responsive. This is feasible, as knock-in lines in which the Cre recombinase is
443 selectively active in MOR-expressing cells are now available[135–137].

444 Achieving higher resolution, Avey and colleagues applied single-cell RNA-seq
445 (scRNA-Seq, using Drop-seq[138]) to analyze transcriptomic changes induced by acute
446 morphine in more than 23,000 cells of the mouse NAc[51]. As is typical in scRNA-Seq,
447 sequencing depth yielded data for ~1,600 genes, corresponding to a reduced genomic
448 representation. Changes were widespread and, interestingly, most frequent in
449 oligodendrocytes, followed by neurons, astrocytes, and microglia, therefore uncovering a
450 cellular hierarchy in transcriptional sensitivity to opioids. More recently, Reiner et al[41] used
451 single-nucleus RNA-Seq (snRNA-seq, 10x Genomics 3') to analyze a larger number of nuclei
452 (190,030) and genes (>2000) in the rat NAc, also following passive and acute morphine
453 injection. Compared to the former study, DEG mostly affected neurons and oligodendrocytes,
454 followed by astrocytes and microglia. Morphine IVSA during 10 days was also investigated,
455 and compared to acute effects. Among DEG shared across the 2 conditions (n=431), the vast
456 majority were dysregulated in a similar direction (up/down), suggesting that they may reflect
457 morphine exposure rather than volitional drug-taking (a "yoked" group chronically exposed to
458 morphine would help substantiate this distinction). Importantly, a larger number of DEG were
459 specifically dysregulated in only one condition (n=868), with more DEG associated with IVSA
460 (reminiscent of the study by Tapocik et al described above), and distinct distributions among
461 NAc cell-types (e.g., Dopamine receptor D1- or D2-expressing neurons). By providing the first
462 single-cell description of morphine IVSA, these results reinforce the notion that voluntary drug-

463 intake may recruit partly distinct molecular mechanisms from those of non-contingent
464 administration.

465 We next compared results on acute morphine from these 2 studies. Both used
466 Seurat[139] and dimensionality reduction (t-SNE[51] or UMAP[41]) to identify clusters, which
467 were then assigned to cell-types using external data. We first compared marker genes
468 reported to define those clusters, when available (Avey: 11/29 clusters; Reiner: 11/19
469 clusters). Low agreement was found (Fig.S4A), with JI ranging from 0 up to 7.1% (for Avey's
470 activated medium spiny neurons, MSNs, and Reiner's D1R MSN-1), possibly because both
471 studies used few marker genes (Avey: 39-51; Reiner: 4-12). We then confronted morphine-
472 induced DEG for each cell-type (Fig.6E): low JI were found, with maximal overlap (6.4%)
473 obtained when comparing Avey's "myelin-forming and mature oligodendrocytes" (MO_all) with
474 Reiner's "Oligodendrocyte-3". Surprisingly, while some clusters assigned to similar cell-types
475 in the 2 studies showed some similarity in DEG (green squares, Fig.6E), strong concordance
476 was also observed across clusters with different identities (e.g. Reiner's "Astrocyte-2" and
477 Avey's MO_all; yellow squares). Overlaps among DEG (Fig.6E) did not increase as a function
478 of cell-type similarity across studies (Fig.S4B), as would have been expected. While a
479 particular emphasis has been put in sc/snRNA-Seq studies on the number of individual cells
480 to be sequenced, these results suggest that in order to maximize their utility: i) marker genes
481 used to assign cellular identities to clusters should be reported comprehensively; and ii) rather
482 than short lists of DEG, differential expression results should be provided for all genes
483 analyzed in each cluster. In addition, classical issues related to sample size, power or
484 statistical models for group comparisons remain pregnant. The Avey and Reiner studies used
485 similar sample sizes (4 and 5 replicates per opioid or control group, respectively), in the upper
486 range of bulk tissue studies (Fig.2D), but applied distinct tools to identify DEG (the SCDE and
487 MAST packages, respectively), which may partly account for discrepancies. With the rapid
488 evolution of this field and emerging controversies (e.g. regarding dimensionality
489 reduction[140]), additional efforts will be necessary to reach consensus on designs and
490 analyses[141, 142].

491

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

493 In parallel to efforts to deepen cellular resolution, others have sought to decipher underlying
494 non-coding RNA and epigenomic regulations[143]. Although neurons are post-mitotic cells,
495 they exhibit peculiar plasticity features that may contribute to the encoding of life events,
496 including exposure to psychoactive drugs[144]. Several non-coding RNA species have been
497 investigated. The first report used custom-made arrays to examine micro-RNA (miR) following
498 morphine or heroin exposure, in rat primary neuronal cultures or the mouse hippocampus[99].
499 7 miR exhibited significant expression changes, among which miR-339-3p was later shown to
500 target the 3'-UTR and down-regulate MOR in response to opioid treatment[145]. A second
501 study used more recent arrays to interrogate all mouse miR in the NAc, following chronic
502 heroin injections[63]. 111 miR exhibited >25% expression changes (no significance reported).
503 The study focused on miR-218, and showed that its over-expression inhibited heroin-induced
504 reinforcement (in conditioned place preference and IVSA), possibly by targeting MeCP2 (a
505 protein that binds methylated DNA), suggesting a potential link between opioids, miR and DNA
506 methylation. Other studies explored long non-coding (lncRNA) or circular RNA (circRNA). One
507 group used 2 arrays interrogating 9,000 lncRNA[93] and 15,000 circRNA[96] to characterize
508 chronic morphine effects in the rat spinal cord: 136 lncRNA (84 up, 52 down; nominal p-
509 value<0.05, |log2FC|>1.5) and 2038 circRNA (896 up, 1142 down; nominal p-value<0.05,
510 FC>2) were affected. Another report used RNA-Sequencing to focus on circRNA in the
511 NAc[64], among which 112 were differentially expressed between mice that had received
512 chronic morphine injections with or without electroacupuncture (51 up, 61 down; nominal p-
513 value<0.05, |log2FC|>1.5). Understanding how these non-coding RNA contribute to OUD will
514 require further work, as numerous mechanisms have been implicated in their physiological
515 effects (including the sequestering of miR by circRNA, or complementarity interactions among
516 lncRNA and mRNA).

517 Regarding the epigenome, a handful of candidate studies had initially provided
518 evidence for differential DNA methylation at specific loci: the MOR gene in human blood[146–

148], and a few loci in the rat brain[149]. At the genome-wide scale, 6 recent studies (1 in rats[62], 5 in humans investigated DNA methylation. The rat study used Whole Genome Bisulfite Sequencing (WGBS) to analyze DNA methylation changes after heroin IVSA, in a standard or enriched environment, in 3 brain regions (VTA, NAc and medial prefrontal cortex)[62]. Because of low sequencing depth, DNA methylation was averaged across the whole genome for selected features (promoters, CG islands). As expected, neither IVSA nor environmental enrichment had any impact on these metrics, suggesting that the methylome is not massively reprogrammed. Opioids may still, however, trigger more discrete DNA methylation changes, consistent with the intuition that the large transcriptomic changes associated with opioid exposure reviewed above are unlikely to occur in the absence of any methylomic plasticity.

Two human studies conducted on bulk brain tissue strengthen this hypothesis. Focusing on Brodmann area 9 (BA9), the first applied EPIC arrays to a cohort of OUD patients (n=19) and controls (n=11)[75]. Among >800,000 CG sites (where DNA methylation mostly occurs in the mammalian brain), 11,917 located at transcription start sites (TSS) showed nominally significant differences (p-value<0.05; unfortunately, genomic localization of these probes, and full genome-wide results, were not provided). Such studies face the difficult task of controlling for confounding factors, as variation in genotype, age, sex, tissue cellular composition or socio-demographic factors have all been associated with DNA methylation changes. The second study addressed these questions[76]. Adjusting for socio-demographic characteristics, ancestry and cellular composition, the authors investigated dorsolateral prefrontal cortex from a large cohort (n=153) of 72 individuals who died of acute opioid intoxication, 53 psychiatric controls, and 28 healthy controls. Although no individual CG site survived multiple testing correction, 13 passed a relaxed significance threshold (p<1.0E-05) for an association with opioid intoxication, one of which was located in Netrin-1, a gene regulating KOR synthesis. At the genome-wide level, opioid use was also associated with enriched differential methylation in the KEGG pathway related to dopaminergic signaling.

While aforementioned studies focused on bulk tissue, 3 others used Fluorescence-Activated Nuclei Sorting (FANS) to achieve cell-type specific analysis of the neuronal methylome, in the orbitofrontal cortex[72–74]. The first used 450K arrays and, comparing 37 heroin users who died from heroin overdose with 28 controls, identified 1298 differentially methylated CG sites, which were more frequently annotated to genes expressed by glutamatergic than GABAergic neurons[73]. The 2 last studies focused on a smaller cohort of male subjects only (12 OUD patients, 26 controls), using Reduced Representation oxidative Bisulfite Sequencing (RRoxBS). Compared to arrays, this allowed for the investigation of a broader set of cytosines (3.5 million CG sites at an average 10X coverage) and, importantly, the characterization of 2 types of non-canonical modifications enriched in the brain: DNA methylation in the non-CG context (also called mCH, where H stands for A, C or T)[150, 151] and hydroxymethylation at CG sites (or hmCG, generated by oxidation of methylated CG, mCG, by *TET* enzymes[152]). Differential methylation events were found to be more frequent in the non-CG (n=2352) than in the reference CG context (n=397), with enrichments in GO terms and gene sets (from Genome-Wide Association Studies, GWAS) related to neuronal function and psychiatric diseases[72]. Similarly, hmCG levels were found more frequently modified (n=1740) than mCG, at least in genomic regions covered by RRoxBS, and enriched in GO terms and co-methylation modules relevant to neuronal physiology[74]. Together, these 2 studies suggest that OUD pathophysiology may be strongly dependent on atypical, brain-enriched forms of methylomic plasticity. Of note, these human studies identified OUD-associated differences at the level of individual cytosines. However, evidence from other research fields and animal models[153, 154] argues for a model whereby DNA methylation changes with functional impact (e.g. on transcription factor binding, or interactions with other epigenetic factors) implicate groups of cytosines in close proximity and with differential methylation in similar direction (differentially methylated regions, DMR), rather than individual sites. Future work should therefore seek to combine DMR calling with the statistical models necessary to account for human confounding factors.

573 Interacting with these DNA modifications, epigenomic plasticity also relies on histone
574 proteins and chromatin structure or accessibility. Surprisingly, a single study characterized
575 histone modifications in the mouse NAc[65]. This pioneering work showed that behavioral
576 effects of chronic morphine (including locomotor sensitization and precipitated withdrawal)
577 were bidirectionally modulated by down- or over-expression of the histone methyltransferase
578 G9a, and accompanied by modulation of H3K9me2 levels across thousands of genomic sites.
579 While these results, and later work on cocaine[152, 155], suggest widespread histone
580 changes, no follow-up work has been published in relation to opioids, to our knowledge.
581 Similarly, only one study has investigated chromatin accessibility. Focusing on a human cohort
582 of 10 heroin users who died from heroin overdose, and 10 healthy individuals[87], genomic
583 regions with "open" chromatin were identified using ATAC-Seq, in the NAc and putamen. The
584 technique was applied to neuronal and non-neuronal cells isolated by FANS. Heroin use was
585 associated with enriched chromatin accessibility in specific gene (CG islands, promoters, 5'
586 untranslated regions) or regulatory (peaks for the H3K4me1 histone mark, binding sites for
587 the EZH2 transcription factor) features, as well as more frequent changes in neurons than in
588 non-neurons (affecting GO terms related to the synapse, among others). At the behavioral
589 level, FYN, a tyrosine kinase prioritized from human data, was then shown in the rat to
590 significantly contribute to heroin IVSA. Overall, this work nicely illustrates how epigenetic
591 analyses may uncover chromatin mechanisms contributing to OUD.
592
593 **Discussion**
594 Here we systematically assessed functional genomic mechanisms of opioid action and OUD,
595 and uncovered a surprisingly low convergence across studies. As a result, a lot remains to be
596 done to move the field forward. Below, we propose recommendations for study design and
597 data sharing, as well as perspectives to refine behavioral modelling, improve cellular
598 resolution, and advance multiomic data integration.
599

600 ***Recommendations for study design and data sharing.*** The low sample sizes observed in
601 the present review (Fig.2D,F) were surprising, considering that this factor is arguably the most
602 important one influencing statistical power[156–158]. Accordingly, a more rigorous analysis of
603 power, and larger sample sizes[159], will lead in future work to better reproducibility, and
604 should be enforced, especially when studying rodent outbred strains, or humans, who display
605 higher inter-individual variability[160, 161]. In humans, similar to what has been observed for
606 GWAS[162], functional genomic studies of OUD are expected to evolve in the next decade
607 towards collaborative efforts involving multiple labs (e.g. the SCORCH consortium[163]), and
608 the progressive aggregation of ever larger cohorts. Another major recommendation relates to
609 data sharing. While public repositories have been specifically implemented for collecting and
610 disseminating omic data (GEO[164], the IHEC data portal[165]) or bioinformatic pipelines
611 (Zenodo, GitHub), their systematic use is not yet current practice in the OUD field, as
612 exemplified in the present work (with similar criticisms raised in other research domains[166]).
613 This hampers re-analysis of published data, comparisons of results across laboratories, and
614 the use of threshold-free methods. We believe this last point is particularly meaningful. Most
615 opioid studies report subsets of DEG identified using arbitrary significance cut-offs, which can
616 mask subtle changes holding relevant biological information (Fig.6[131]). We therefore
617 strongly advocate for generalized use of threshold-free algorithms for enrichment analysis of
618 single datasets (GSEA), or comparison across pairs of differential analysis results (RRHO2).
619 Within this line, newer packages are expected to scale-up the applicability of such tools to
620 epigenomic data, which frequently involve larger number of observations than gene-level
621 counts[167].
622
623 ***Refine behavioral modeling.*** The face validity of SA or oICSS models of OUD has
624 significantly increased over last 2 decades[168], and we now face the task of more
625 systematically combining them with most recent molecular approaches. Another important
626 step will be to decipher the molecular mechanisms that may differentially account for distinct
627 behavioral dimensions, among impulsive or persistent drug-seeking, resistance to

628 punishment, or excessive motivation (including those modelled using passive administration,
629 such as tolerance, sensitization, or withdrawal). While the vast majority of studies reviewed
630 here used a categorical comparison of animals exposed or not to opioids, another design,
631 based on a dimensional approach within the exposed group, could help address the inter-
632 individual variability in susceptibility or resilience to OUD. The latter design has been used by
633 only one article in the present review[56] (section *2.1*), which contrasts with the amount of data
634 available for models of other psychiatric disorders, in particular depression (e.g. chronic social
635 defeat[169–171]. To better understand such inter-individual susceptibility, epigenetic
636 mechanisms are obvious candidates[172], as illustrated by 2 recent studies on DNA
637 methylation and oral SA of alcohol in macaques[173], and miRNA in a rat model of cocaine
638 SA[174]. While similar mechanisms are likely at play in OUD, they have not been addressed
639 by open-ended approaches yet, pointing toward an important avenue for future work.
640
641 ***Improve cellular resolution***. This is another major avenue for future work, considering the
642 extreme heterogeneity of brain tissue. In addition to cell-type specific (TRAP) and sc/snRNA-
643 Seq (already applied to OUD, see above), a newer generation of tools now enables similar
644 molecular profiling while taking neuroanatomical context into consideration, with 2 possible
645 factors: i) connectivity patterns, probed using antero- or retrograde viruses, in combination
646 with TRAP (transcriptomics[175]) or FANS (epigenomics[176]); or ii) spatial context, with
647 methodologies based on sequencing or microscopy to detect RNA "in histological space";
648 while spatial capture followed by transcriptome sequencing (e.g. Slide-seq[177]) enables
649 genome-wide analysis with 10µm resolution, single-molecule fluorescence in situ hybridization
650 (smFISH) allows for better spatial resolution (1µm), but requires probe design and target gene
651 selection[146,147] (covering a few hundred to a few thousand genes, lower than sc/snRNA-
652 Seq[180]). Future work using these techniques, by characterizing molecular effects of opioids
653 in a particular neuronal pathway or cell-type, or with exquisite spatial resolution, should
654 deepen the understanding of OUD.
655

656 ***Advance multiomic data integration***. The epigenomic and transcriptomic mechanisms
657 reviewed above contribute to changes at protein level that ultimately mediate
658 neurophysiological adaptations. Proteomic studies were initially not included in our systematic
659 bibliography, considering the low number of studies available and because, compared to RNA-
660 or DNA-sequencing methodologies, they only cover a few hundred proteins[181–184]).
661 Nevertheless, to go further in the understanding of OUD, future work will need to combine
662 transcriptomic and proteomic analyses with the characterization of upstream epigenetic
663 regulation. The next challenge, therefore, will be to integrate those various molecular layers
664 into multiomic models of pathophysiology.

665 Illustrating these concepts, we recently conducted a human study suggesting that
666 early-life adversity and depression associate with 2 forms of DNA methylation changes in the
667 CG and non-CG contexts (specifically focusing on CAC sites, where non-CG methylation is
668 most abundant)[150]. By combining WGBS with ChIP-Seq analysis of 6 histone marks, the 2
669 forms of plasticity were found to affect distinct genomic sites and features, and to be defined
670 by different histones modifications and chromatin states. Similar multiomic investigations of
671 opioid effects should be conducted and, importantly, will require dedicated bioinformatic
672 frameworks, which are only emerging in psychiatry[185]. Only 2 studies reviewed here
673 followed that path: Mendez et al performed transcriptomic and proteomic analyses, while Liu
674 et al analyzed the transcriptome and methylome (see *4.Non-coding and epigenomic*
675 *mechanisms of opioid plasticity*). In both studies, multiomic integration consisted: first, in the
676 identification of features (single mRNA, DNA methylation site, or protein) passing a
677 significance threshold, and their overlap; second, in the construction of co-expression or co-
678 methylation networks, and the prioritization of groups of features, defined as modules, most
679 significantly associated with OUD, and their biological interpretation (enrichment in functional
680 GO terms, significantly affected features, GWAS results, etc). While such systems-biology
681 approaches will undoubtedly contribute to the understanding of OUD, they remain based on
682 step-by-step aggregation of results generated individually for each layer. In the coming years,
683 it is expected that advanced multiomic integration approaches (e.g. similarity network fusion,

684 or multiblock methods[186–188]), may more globally define, throughout the genome, how
685 multiple regulatory layers interact, and the chain of events linking genetic, developmental, and
686 environmental factors to abnormal behavior and compulsive drug use[189].
687
688 **Conclusion**
689 Although numerous functional genomic studies of opioid action and OUD have been
690 conducted across animal models and patient cohorts, replicable findings appear limited. In the
691 future, combining refined behavioral models with multiomic integration, at the single-cell scale,
692 will hopefully pave the way towards the identification of innovative therapeutic targets and,
693 ultimately, contribute to better care.
694
695

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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.

715 **Figure legends**

716 **Figure 1. PRISMA diagram.** The review protocol was registered prospectively in Prospero
717 (record ID#CRD42022270113), and followed the Preferred Reporting Items for Systematic
718 Review and Meta-Analysis protocol (PRISMA[190]). ***Identification of studies***. Three
719 databases were used for systematic screening: MEDLINE, Embase and Web of Science. The
720 literature search was performed using the following keywords: “microarray” OR “RNA
721 sequencing” OR “bisulfite sequencing” OR “chromatin immunopurification sequencing” OR
722 “single cell RNA sequencing” AND each of the following terms: “opiates”, “opioid”, “morphine”,
723 “fentanyl”, “oxycodone”, “heroin”, “methadone” or “buprenorphine”. The initial search provided
724 2709 potentially eligible studies. ***Screening***. Articles were screened based on their title and
725 abstract, and included if they used a high-throughput, genome-wide methodology to assess
726 modifications of gene expression or epigenetic mechanisms as a function of opioid exposure,
727 in the nervous system of primates or rodents (rat, mouse), and were published in English in
728 peer-reviewed journals. Exclusion criteria included tissue other than the nervous system and
729 candidate genes approaches. Once duplicates were removed, a total of 1229 articles were
730 screened, among which 1067 were excluded because their title or abstract didn’t meet
731 eligibility criteria. ***Eligibility***. After a more thorough examination of articles’ full-text, an
732 additional 89 articles were excluded. As a result, 73 papers were selected for qualitative
733 synthesis. Among these, 44 articles reporting on transcriptomic analyses in the 5 most
734 frequently investigated regions were selected for a more detailed quantitative synthesis. Of
735 these, 34 articles investigated a single brain region, 9 covered 2 regions, and only 1
736 characterized 5 regions [13]. Overall, this resulted in 52 differential expression analyses: 18,
737 11, 11, 6 and 6 in the nucleus accumbens (NAc), frontal cortex, whole striatum, dorsal striatum
738 and spinal cord, respectively.

739

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

743 sizes in rodent and human studies that investigated both sexes and compared opioid-exposed
744 animals to controls (rodents, panel E), or individuals with a diagnosis of opioid use disorder
745 (OUD) to healthy controls (humans, panel F).
746
747 **Figure 3. Characteristics of studies included: Injection parameters, Brain structure &**
748 **Techniques.** Among the 73 studies considered during qualitative analysis, a large panel of
749 injection parameters, nervous system structures and techniques were represented. **(A)**
750 Injection parameters used for opioid administration. NB: "mixed" refers to a single study that
751 used a combination of chronic oral administration (through bottles) and acute ip injections of
752 morphine[79]. **(B)** Number of articles investigating each nervous system structure. **(C)** Side-
753 by-side comparison of the number of articles investigating each structure with the protein
754 expression profile of the molecular target of opioids, the mu opioid receptor (MOR; as
755 assessed using [^{3}H]-DAMGO autoradiography, data in fmol/mg of tissue, adapted from[115,
756 118–120]). **(D)** Distribution of high-throughput methodologies and year of publication across
757 studies. *Abbreviations:* FCx, Frontal cortex; NAc, Nucleus accumbens; dStr, Dorsal Striatum;
758 HT, Hypothalamus; Th, Thalamus; mHb, Medial Habenula; Hc, Hippocampus; Amy,
759 Amygdala; VTA, Ventral Tegmental Area; PAG, periaqueductal gray; DRN, Dorsal Raphe
760 Nucleus; LC, Locus Coeruleus; NTS, Nucleus Tractus Solitarius.
761
762 **Figure 4. Data availability for eligible studies and quantitative analysis in the nucleus**
763 **accumbens. (A)** The graph depicts the number of: i) differential expression analysis results
764 considered for quantitative analysis (with the corresponding number of articles between
765 brackets); ii) cases when lists of differentially expressed genes (DEG) were available; iii) cases
766 when full genome-wide results were available (with both fold changes, FC, and p-values). **(B)**
767 Number of DEG identified in studies that investigated the nucleus accumbens. **(C-E)** Venn
768 diagram of DEG identified in each species (human, mice and rat). **(F)** Venn diagram of DEG
769 in the 2 studies that investigated the dorsal striatum.
770

771 **Figure 5. Quantitative analyses in the dorsal striatum, whole striatum and frontal cortex.**
772 **(A)** Number of differentially expressed genes (DEG) identified in studies that investigated the
773 striatum (see Fig.S1 for separate analyses of studies that used acute or chronic opioid
774 administration). **(B)** Number of DEG in studies of the frontal cortex. **(C)** Number of genes
775 identified as differentially expressed (DE) across different studies. **(D)** Across the 5 brain
776 regions considered during quantitative analysis, we assessed the impact of the following 7
777 factors on consistency among studies, using pairwise Jaccard indexes: species, brain
778 structure, treatment chronicity (acute/chronic), opioid drug (morphine/heroin), strain
779 (C57BL6J/others), time of analysis following last exposure (5 lists of DEG from tissue collected
780 1 hour after opioid exposure, 5 after 2 hours, 10 after 4 hours, 4 after 8 hours, 1 after 14 hours,
781 and 3 after 24 hours) and article. For each factor (e.g., species), pair-wise JI were classified
782 in 2 categories describing whether they belonged to the same sub-group (e.g., 2 studies
783 conducted in humans; "intra"), or not (e.g., 1 study in humans compared to another in rats;
784 "inter"), within that factor. For the drug factor, studies in human were not considered as they
785 cannot be assigned to specific opioids; a single study investigated fentanyl, and was
786 excluded[33]. For the strain factor, among the 14 mouse studies available, JI significantly
787 increased among the 12 that used C57BL6J mice, while a similar analysis was not possible
788 for 129P3/J, DBA/2J, SWR/J or Kunming strains, as only 1 list of DEG was available for each.
789 One study reporting on the combined analysis of C57BL6J and DBA2J mice was excluded[54].
790 No similar analysis was possible in rats, as all available studies used Sprague-Dawley (except
791 one that did not state the strain used[68]). Note that the numbers of JI values considered in
792 each comparison are indicated at the bottom of each bar.
793
794 **Figure 6. (A-D) Threshold-free comparison of 2 bulk tissue RNA-sequencing studies**
795 **(Seney et al and Townsend et al), and cell-type specific comparison of 2 single-cell**
796 **(Avey et al) and single-nucleus (Reiner et al) RNA-sequencing studies. (A-B)** Venn
797 diagram of differentially expressed genes (DEG) identified across the 2 bulk tissue RNA-
798 Sequencing studies by Seney et al and Townsend et al (DEG defined by arbitrary significance

799 thresholds in each study: p-value < 0.05 for both) for women/female rats (A) and men/male
800 rats (B). **(C-D)** Genome-wide, threshold-free comparison of the same 2 studies using Rank-
801 Rank Hypergeometric Overlap (RRHO2) for women/female (C) rats and men/male rats (D). In
802 RRHO2, briefly, genes are ranked using p-values (signed according to directionality), from
803 upregulated (Seney: left, Townsend: bottom) to down-regulated genes (Seney: right, fentan:
804 top). Overlaps along the 2 distributions are then iteratively assessed using hypergeometric
805 testing. High p-values in the bottom-left and top-right quadrants indicates concordance,
806 whereas high p-values in the other 2 quadrants (bottom-right and top-left) indicate discordance
807 between the 2 datasets. The white crosses indicate the location of the overlap corresponding
808 to Venn diagrams depicted in panels A-B (split in subgroups of up- or down-regulated genes),
809 while black crosses correspond to most significant overlaps identified using RRHO2. **(E)**
810 Heatmap of pairwise Jaccard indexes for DEG identified across each of the 26 and 27 cell-
811 types defined by Avey et al and Reiner et al, respectively.
812

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1304

Figure 1

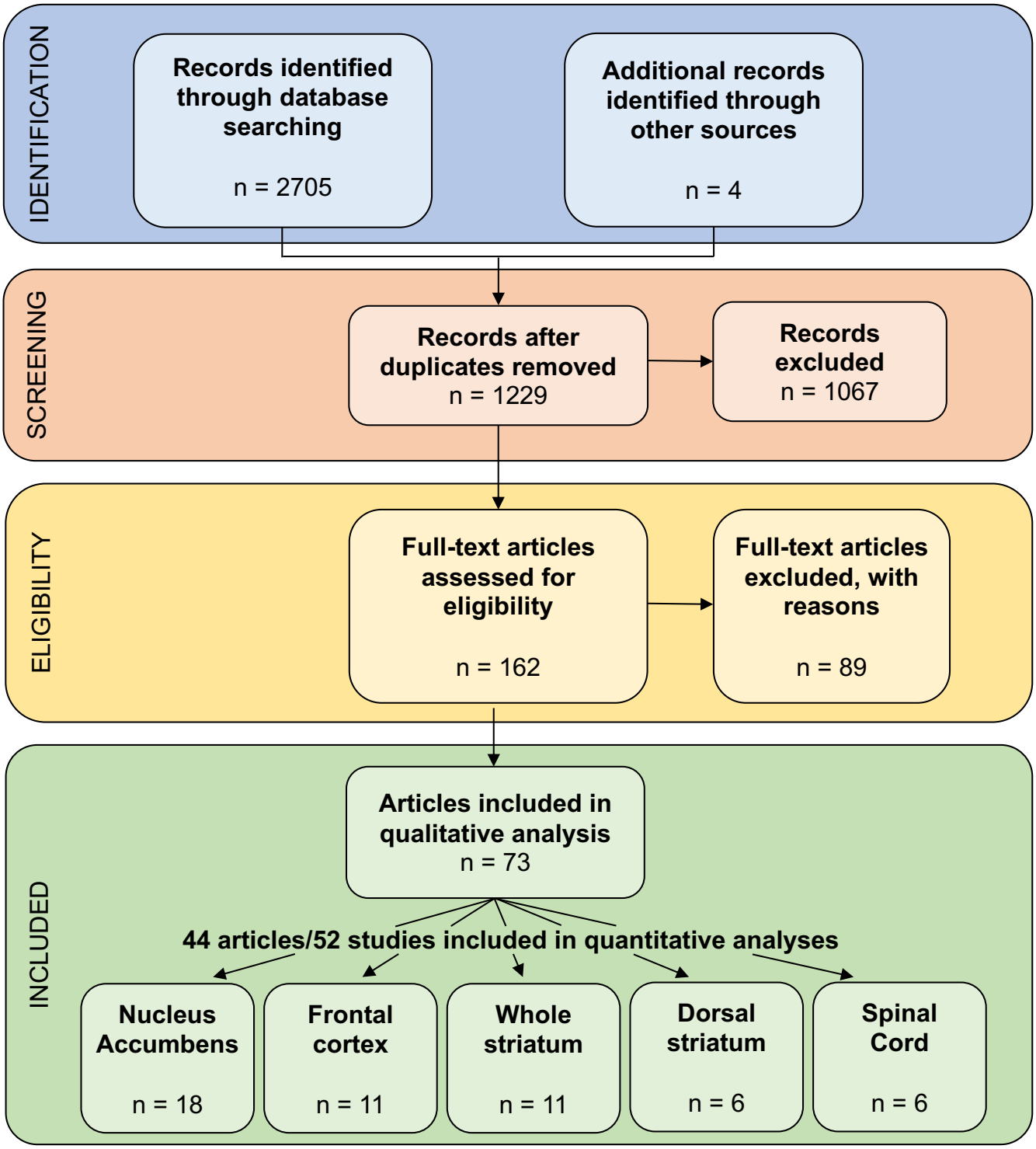

IDENTIFICATION
Records identified through database searching
n = 2705
Additional records identified through other sources
n = 4
SCREENING
Records after duplicates removed
n = 1229
Records excluded
n = 1067
ELIGIBILITY
Full-text articles assessed for eligibility
n = 162
Full-text articles excluded, with reasons
n = 89
INCLUDED
Articles included in qualitative analysis
n = 73
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

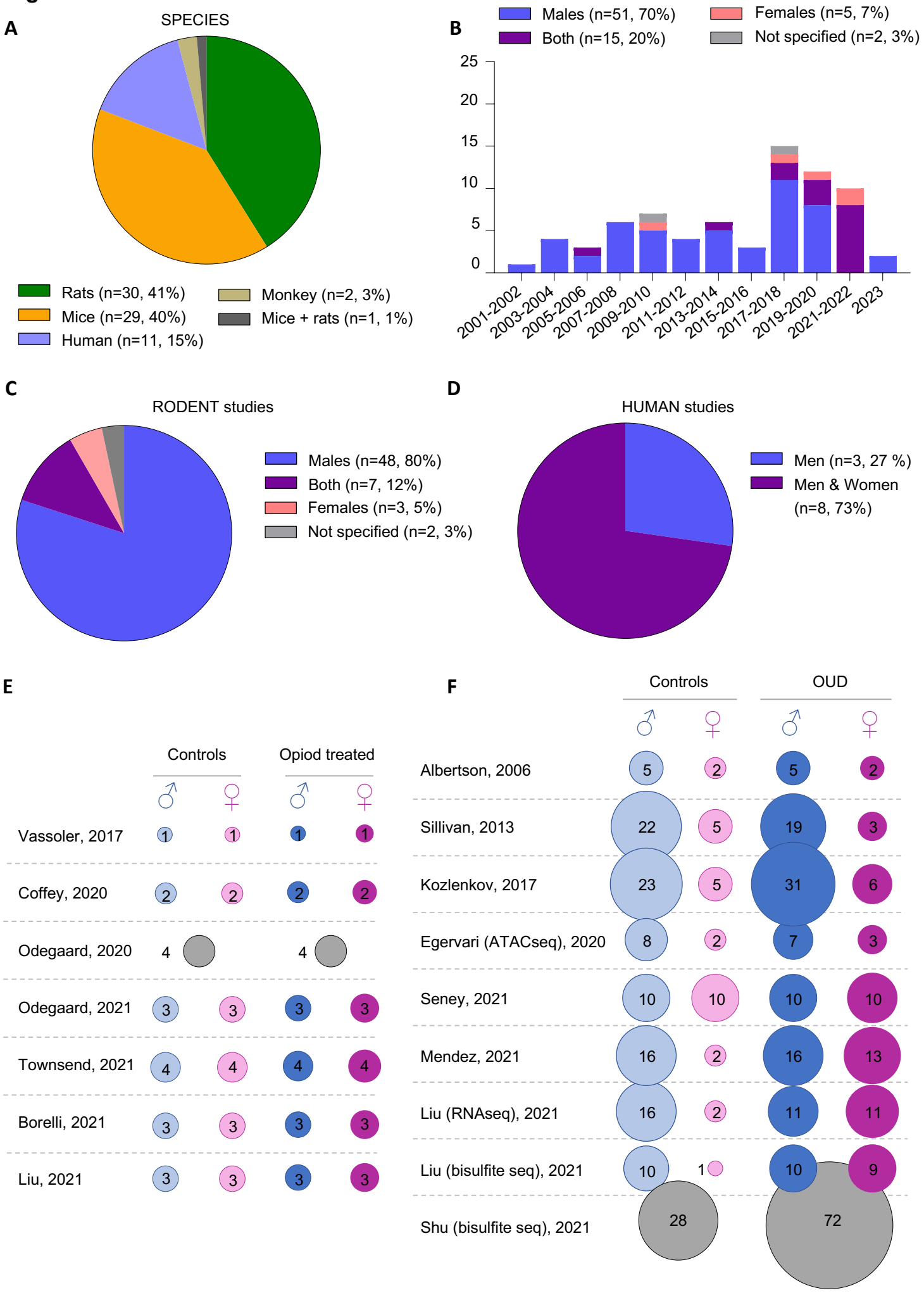

A
SPECIES
Rats (n=30, 41%)
Mice (n=29, 40%)
Human (n=11, 15%)
Monkey (n=2, 3%)
Mice + rats (n=1, 1%)
B
Males (n=51, 70%)
Both (n=15, 20%)
Females (n=5, 7%)
Not specified (n=2, 3%)
25
20
15
10
5
0
2001-2002
2003-2004
2005-2006
2007-2008
2009-2010
2011-2012
2013-2014
2015-2016
2017-2018
2019-2020
2021-2022
2023
C
RODENT studies
Males (n=48, 80%)
Both (n=7, 12%)
Females (n=3, 5%)
Not specified (n=2, 3%)
D
HUMAN studies
Men (n=3, 27 %)
Men & Women (n=8, 73%)
E
Controls
Opiod treated
Vassoler, 2017
Coffey, 2020
Odegaard, 2020
Odegaard, 2021
Townsend, 2021
Borelli, 2021
Liu, 2021
F
Controls
OUD
Albertson, 2006
Sillivan, 2013
Kozlenkov, 2017
Egervari (ATACseq), 2020
Seney, 2021
Mendez, 2021
Liu (RNAseq), 2021
Liu (bisulfite seq), 2021
Shu (bisulfite seq), 2021

Figure 3

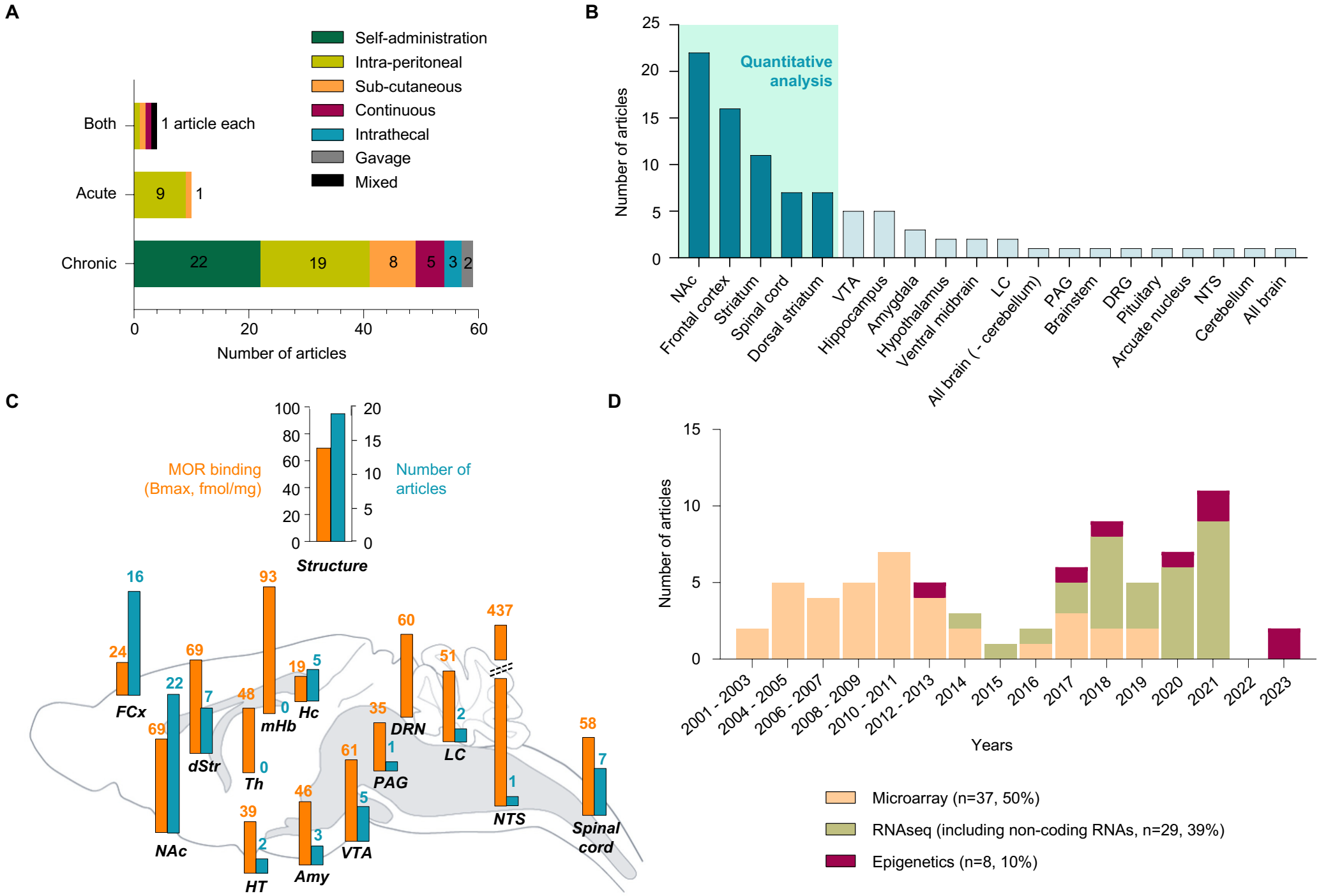

A
Self-administration
Intra-peritoneal
Sub-cutaneous
Continuous
Intrathecal
Gavage
Mixed
Both
1 article each
Acute
9
1
Chronic
22
19
8
5
3
2
0
20
40
60
Number of articles
B
Quantitative analysis
Number of articles
25
20
15
10
5
0
NAc
Frontal cortex
Striatum
Spinal cord
Dorsal striatum
VTA
Hippocampus
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
NAc
dStr
HT
mHb
Th
Hc
Amy
VTA
PAG
DRN
LC
NTS
Spinal cord
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
2022
2023
Microarray (n=37, 50%)
RNAseq (including non-coding RNAs, n=29, 39%)
Epigenetics (n=8, 10%)

Figure 4

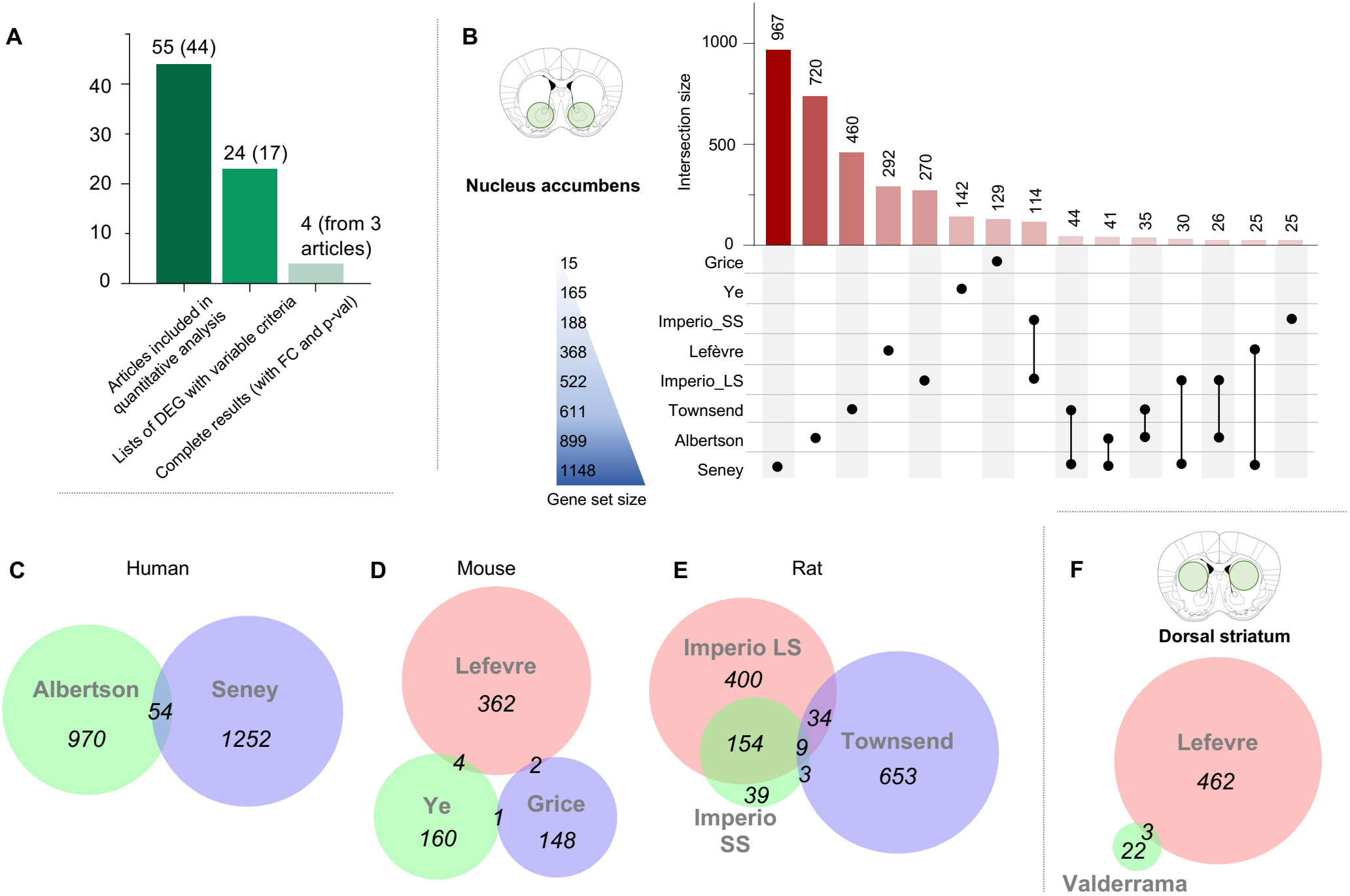
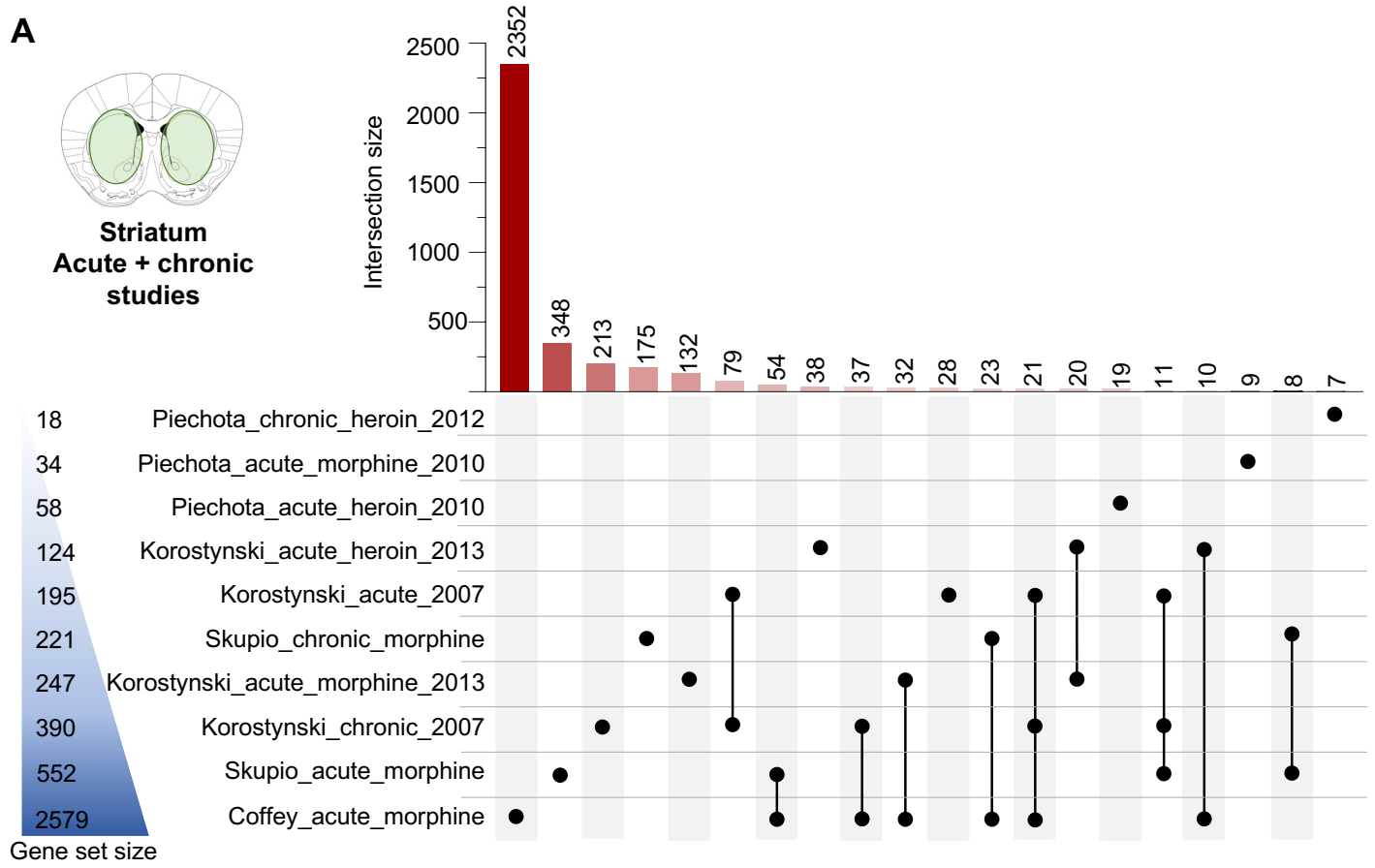
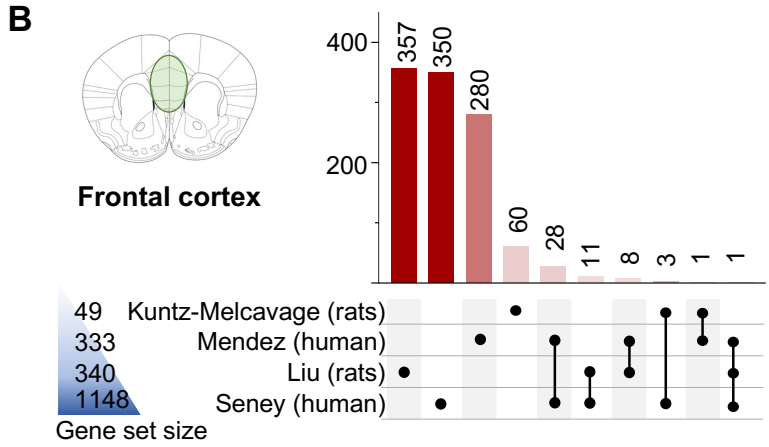
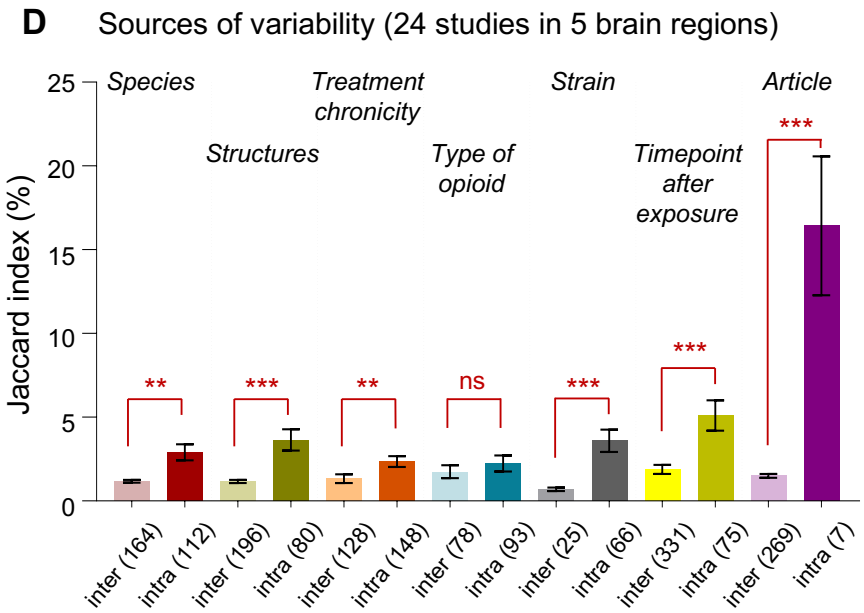

A
55 (44)
24 (17)
4 (from 3 articles)
0
10
20
30
40
Articles included in quantitative analysis
Lists of DEG with variable criteria
Complete results (with FC and p-val)
B
Nucleus accumbens
Intersection size
0
500
1000
967
720
460
292
270
142
129
114
44
41
35
30
26
25
25
Grice
Ye
Imperio_SS
Lefèvre
Imperio_LS
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
2500
2000
1500
1000
500
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
400
200
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


D
Sources of variability (24 studies in 5 brain regions)
Jaccard index (%)
25
20
15
10
5
0
Species
Treatment chronicity
Strain
Article
Structures
Type of opioid
Timepoint after exposure
**
***
**
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)

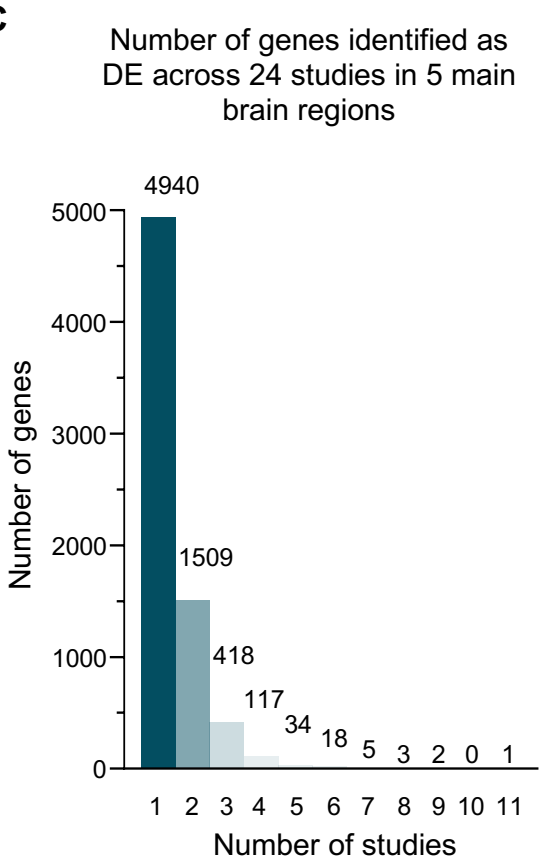
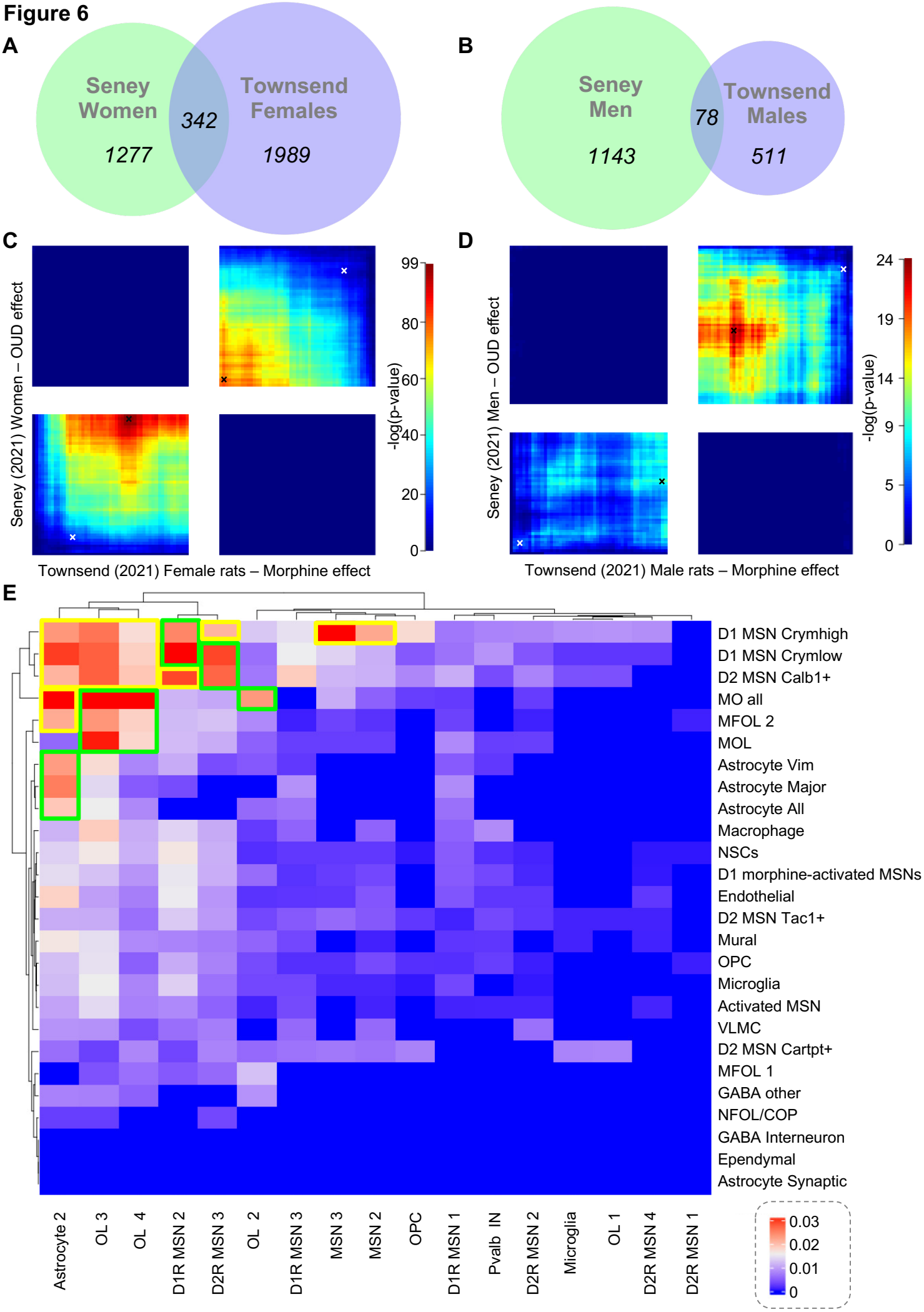

C
Number of genes identified as DE across 24 studies in 5 main brain regions
Number of genes
5000
4000
3000
2000
1000
0
4940
1509
418
117
34
18
5
3
2
0
1
1 2 3 4 5 6 7 8 9 10 11
Number of studies

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