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Molecular tools to elucidate factors regulating alcohol use

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Abstract

Alcohol use disorders (AUD) are pervasive societal problems, marked by high levels of alcohol intake and recidivism. Despite these common disease traits, individuals diagnosed with AUD display a range of disordered drinking and alcohol-related behaviors. The diversity in disease presentation, as well as the established polygenic nature of the disorder and complex neurocircuitry, speak to the variety in neurochemical changes resulting from alcohol intake that may differentially regulate alcohol-related behaviors. Investigations into the molecular adaptations responsible for maladaptive alcohol-related behavioral outcomes require an ever-evolving set of molecular tools to elucidate with increasing precision how alcohol alters behavior through neurochemical changes. This review highlights recent advances in molecular methodology, addressing how incorporation of these cutting-edge techniques not only may enhance current knowledge of the molecular bases of AUD, but also may facilitate identification of improved treatment targets that may be therapeutic in specific subpopulations of AUD individuals.

Keywords

RNAi; TRAP; FLEX; CRISPR

Introduction

Alcohol use disorder (AUD) is a chronic condition characterized by loss of control over alcohol intake and high levels of relapse in spite of individuals' desire to maintain sobriety. The progressive and pervasive nature of this disorder, with escalation of alcohol intake over time and chronic recidivism, suggests alcohol consumption generates neuroadaptations that promote high alcohol drinking and persist long after the cessation of alcohol intake. AUD is a complex mental illness encompassing multiple disease phenotypes (Salvatore, Gottesman,

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Conflict of interest

The author has no conflicts to report.

& Dick, 2015) and genotypes (Hart & Kranzler, 2015) under a common diagnosis, with low penetrance of individual mutations across the AUD population. The polygenic nature of AUD not only contributes to behavioral variation within the diagnosis, but also likely restricts the efficacy of any individual pharmacological target to treat all AUD individuals. Together, these features implicate a variety of molecular neuroadaptations that may support divergent patterns of alcohol abuse and relapse. Systematic elucidation of the molecular changes resulting from alcohol consumption, as well as determination of their functional relevance, is crucial not only to provide greater knowledge about mechanisms underlying disordered alcohol use, but also to identify novel medication targets with the greatest potential to treat subsets of AUD patients. For treatments to succeed, however, understanding the specificity of individual molecular changes at the neuronal, brain region and circuit levels, as well as their functional impact on behavior, is essential. This review addresses the evolution of methods to investigate and manipulate the molecular composition of neurons with increasing precision, with a focus on how recent advances in molecular tools may enhance our current state of understanding of the molecular bases of AUD.

Ribosome-directed technologies for profiling alcohol's molecular impact on neurons

Identifying the vast number of molecular adaptations triggered by different patterns of alcohol consumption is crucial for complete understanding of the biochemical bases of AUD, which may yield novel directions for treatment development. As the template for new protein translation, mRNA expression has long been a primary measure of molecular neuroadaptation to alcohol exposure. Over the two decades since its development significantly improved the quantitative precision of mRNA measurement, real-time quantitative polymerase chain reaction (qPCR) (Heid, Stevens, Livak, & Williams, 1996) has greatly enhanced our understanding of alcohol's impact on brain neurochemistry. Specifically, qPCR analysis of total mRNA content in microdissected brain tissue has allowed precise determination of brain region-specific changes in transcript expression for neuropeptides and receptor systems pharmacologically implicated in the regulation of alcohol drinking, as well as identifying novel factors involved in controlling alcohol intake. One pharmacologically identified neuropeptide system validated by qPCR as altered by excessive alcohol exposure was the corticotropin-releasing factor (CRF)/urocortin system. Repeated bouts of alcohol intoxication via chronic intermittent vapor exposure or binge-like drinking conferred sensitivity to CRF type 1 receptor (CRF₁) antagonist regulation of drinking (Funk, O'Dell, Crawford, & Koob, 2006; Valdez et al., 2002). This enhanced antagonist sensitivity likely resulted from CRF system upregulation, since both receptor (*Crhr1*) and ligand (*Crh*) mRNA levels were increased in the central amygdala (CeA), as were the alternate ligand, urocortin-1 (*Ucn1*), in the centrally-projecting Edinger Westphal nucleus (Giardino et al., 2017; Lack, Floyd, & McCool, 2005; Roberto et al., 2010; Sommer et al., 2008). In contrast to these neuroadaptations promoting alcohol drinking, brain region-specific analysis of total mRNA by qPCR also has identified genes acutely upregulated by alcohol to protect against the development of disordered alcohol drinking. These include brain-derived neurotrophic factor (*Bdnf*), increased in the dorsal striatum after alcohol injection, moderate intake (McGough et al., 2004), or even a single bout of drinking (Logrip, Janak, & Ron, 2009), and glial-derived neurotrophic factor (*Gdnf*), elevated in the ventral tegmental area (VTA) and nucleus accumbens (NAc) after moderate alcohol exposure

(Ahmadiantehrani, Barak, & Ron, 2014; Barak et al., 2015). These are but a few examples of the myriad molecular systems contributing to the regulation of alcohol intake whose total mRNA expression levels were quantified via qPCR with brain region-specific precision.

Polysomal mRNA purification—Despite its quantitative advantage over mRNA abundance assessment by traditional PCR, qPCR analysis of total mRNA expression suffers from several drawbacks addressed by recent technological advances to improve measurement of behaviorally relevant gene expression. First, although up- or down-regulation of mRNA transcription is customarily the first step toward altering the protein composition of the neuron, changes in total mRNA expression do not uniformly align with modifications in protein levels (Y. Liu, Beyer, & Aebersold, 2016). This mRNA-protein mismatch may result from posttranscriptional events affecting mRNA translation, such as microRNA inhibition of translation of newly transcribed mRNA sequences into protein products (Smith & Kenny, 2017). Conversely, changes in mRNA abundance that are cell type- or neuronal compartment-specific may go undetected in the mixed-cell, whole-neuron mRNA preparations used for traditional qPCR analyses. Addressing the issue of transcription/translation mismatch requires the quantification of mRNA sequences definitively targeted for translation based on their association with ribosomes. Isolation of polyribosome-associated mRNA (polysomes), purified by differential centrifugation (Lou, Baser, Klussmann, & Martin-Villalba, 2014), provides one mechanism for restricting mRNA quantification to those sequences bound for translation. Analysis of NAc polysomal mRNA from mice or rats with a history of binge-like alcohol drinking identified two novel alcohol-regulated gene targets: *Crmp2* (collapsin response mediator protein-2) and *Prosapip1* (Laguesse et al., 2017; F. Liu et al., 2017). Both novel targets are downstream of mammalian target of rapamycin (mTOR) signaling, which is also activated by alcohol (Neasta, Ben Hamida, Yowell, Carnicella, & Ron, 2010). Importantly, neither effector differed in expression at the total mRNA level, demonstrating the importance of considering ribosome-associated mRNA as a more accurate measure of nascent protein production.

Ribosome purification via protein tagging: TRAP—While these studies highlight the need to consider the relevance of observed mRNA changes to neuronal function, since the composition of total and translation-targeted mRNA populations may differ, sucrose gradient purification of polysomes is labor intensive and lacks the capacity to restrict analyses by cell type or subcellular compartment. To enhance specificity in translational profiling, Translating Ribosome Affinity Purification (TRAP) was developed as a means for efficient sequestration of ribosomes and their affiliated mRNA sequences (Doyle et al., 2008; Heiman, Kulicke, Fenster, Greengard, & Heintz, 2014) (Figure 1). TRAP relies on the expression of a labeled ribosomal subunit, with the ribosome tag serving as an immunogen for immobilization of ribosomes on magnetic beads via immunoprecipitation. Two varieties of labeled are currently in use: ribosomal protein L10a bearing an EGFP tag (L10a^{EGFP}) (Doyle et al., 2008; Heiman et al., 2008) and HA-tagged ribosomal protein 22 (Rpl22^{HA}, also known as RiboTag) (Sanz et al., 2009). TRAP isolation of ribosome-associated mRNA provides significant benefits over not only total mRNA isolation but also polysomal mRNA, as the TRAP technology can be tailored to isolate specific cellular subpopulations, thereby allowing for cell type-specific mRNA expression profiling. Initial development of TRAP

mice employed the GENSAT bacterial artificial chromosome (BAC) transgenic mice with known cell type-specificity of BAC expression, yielding multiple bacTRAP mouse lines with L10a^{EGFP} expression restricted to specific cell subpopulations in each line (Doyle et al., 2008). Developed in parallel, the RiboTag mouse was the first to utilize Cre recombinase-driven incorporation of labeled Rpl22^{HA} ribosomal proteins (Sanz et al., 2009). Cre/loxP expression systems modify gene expression in a targeted manner via the incorporation of loxP sites flanking a sequence of interest. In the presence of Cre recombinase, loxP sites recombine to excise or invert a loxP-flanked sequence, depending on loxP orientation in the transgene (Brault, Besson, Magnol, Duchon, & Herault, 2007), which Sanz and colleagues (2009) employed to replace the endogenous exon 4 of *Rpl22* with an HA-tagged version of the exon. A similar approach was subsequently applied to produce Cre-driven L10a^{EGFP} expression (J. Liu et al., 2014). As Cre recombinase is required for loxP-driven excision/inversion events to occur, restriction of Cre expression according to brain region, cell type or temporal expression profile provides a mechanism for controlling specificity of transgene expression. This technology is poised to greatly refine our understanding of alcohol's neuromodulatory effects, allowing for cell type- or circuit-specific expression of TRAP markers. Elucidation of subpopulation differences in gene expression profiling has the potential to elucidate sources of failure for treatments designed to combat molecular adaptations identified in mixed neuronal populations. For example, our understanding of transcriptional changes in striatal subdivisions would be enhanced not only by comparison of total to translation-targeted mRNA, but in particular by parallel assessment of alcohol remodeling of dopamine D1 vs. D2 receptor-expressing striatal subpopulations. As previously demonstrated in the NAc for cocaine-regulated targets (Chandra et al., 2017; Chandra et al., 2015), alcohol might generate opposing adaptations of the same gene in D1- vs. D2-expressing neurons. This possibility raises significant questions about whether targets previously deemed alcohol-nonresponsive in total mRNA analyses of mixed cell populations must be reassessed at the single cell type level.

One challenge for widespread use of TRAP technology has been the necessity for ribosomal tag expression to enable ribosomal capture, as this has largely limited investigations to transgenic mice (Doyle et al., 2008; Sanz et al., 2009). However, as TRAP methodology has evolved, procedural refinements have expanded the use of TRAP in some very exciting directions. Viral TRAP constructs have been generated for use in Cre-expressing neuron populations (Nectow et al., 2017), providing researchers a means for applying TRAP technology to existing rodent lines expressing Cre in a cell type-restricted fashion without needing to breed new bi-transgenic Cre-TRAP mouse lines for each desired cell type. Expanding translation-targeted mRNA analysis to the circuit level, several methods have been developed to restrict ribosomal tagging by projection target. The first approach employed tagging of ribosomal protein L10a with anti-GFP nanobodies that can bind retrogradely-transported GFP (Ekstrand et al., 2014). This technology was subsequently expanded for adeno-associated viral delivery of Cre-regulated L10a nanobody expression in combination with retrograde GFP virus co-infusion (Nectow, Ekstrand, & Friedman, 2015), an advance allowing not only projection-specific but also cell type-specific expression profiling, based on restriction of Cre expression. Further refinement of retrograde delivery technology using pseudorabies virus for GFP delivery now allows trans-synaptic ribosomal

tagging, such that mRNA expression may be profiled across two or more synapses retrograde to the site of GFP infusion, provided the neurons express Cre recombinase and L10a with anti-GFP nanobodies (Pomeranz et al., 2017). Alternatively, for anterograde circuit assessments, viral TRAP may be combined with retroviral Cre delivery in a defined projection target to measure ribosome-associated mRNA isolated within a specific efferent circuit (Tervo et al., 2016). In addition to refining methods for Cre-mediated TRAP expression, the technology has been modified to remove the requirement for Cre-directed expression, with the development and *in vivo* validation of viral expression constructs that can generate functional, labeled ribosomes in the absence of Cre (Cook-Snyder, Jones, & Reijmers, 2015). Thus circuit-level mRNA expression profiling can be performed either in transgenic rodents expressing Cre in a cell type-restricted fashion or in outbred rodents without Cre-directed cell specificity.

As detailed above, a key advantage of TRAP technology is the ability to narrow down transcriptional investigation to circumscribed populations or regions, including neuronal sub-compartments. Given sufficient physical distance between TRAP-labeled cell bodies and the termini of their axonal projections, axonal ribosome-associated mRNA expression can be quantified through a procedure termed Axon-TRAP (Shigeoka et al., 2016). This allows for comparative profiling of mRNA adaptation across a TRAP-labeled brain region's various terminal fields. Conversely, combining TRAP with synaptosomal fractionation, a procedure termed SynapTRAP, allows researchers to profile ribosome-associated mRNA localized near the postsynaptic density (Ouwenga et al., 2017), likely sources of new proteins for the synaptic remodeling that underlies synaptic plasticity (Bramham, 2008). Together these studies demonstrate the power of TRAP as a means of profiling cell-specific and neuronal compartment-restricted changes in mRNA expression in a translationally relevant milieu, which can refine our assessment of both known and currently unknown alcohol-induced neuroadaptations. Despite the potential for improved precision in molecular investigations afforded by TRAP technology, such analyses only provide snapshots of the molecular state of neurons at the point of tissue collection, showing association but not causation between adaptations and alcohol exposure history. Determining the functional involvement of identified alcohol-responsive targets to regulate alcohol intake requires implementation of methods to manipulate gene expression.

Viral regulation of neuronal effector expression: RNAi, FLEX and CRISPR

Quantitative profiling of mRNA or protein content provides essential knowledge about alcohol-induced changes in brain neurochemistry. Elucidation of the role specific genes and their protein products play in shaping alcohol-related behaviors, i.e. the functionality of the observed neuroadaptations, is a crucial component of novel treatment discovery. Demonstration of causality requires *in vivo* manipulations of gene/protein expression, a central facet of molecular research in the alcohol field since the advent of transgenic technology and its use to generate genetically altered mice (Gordon & Ruddle, 1981; Gordon, Scangos, Plotkin, Barbosa, & Ruddle, 1980; Jaenisch, 1976). Transgenic mouse models have provided a means to ascertain the necessity of specific molecular targets for alcohol-related behaviors over several decades. However, conventional transgenic models lack temporal, regional and cell type specificity, and thus may produce behavioral effects via

developmental adaptations, rather than through acute effects of the protein's absence, or conversely may fail to alter behavior due to developmental compensation or contrasting effects of the mutation in different cell types. While refinement of transgenic techniques have improved on some of these shortcomings, particularly through the introduction of Cre-mediated gene deletion (Gu, Zou, & Rajewsky, 1993; Schwenk, Baron, & Rajewsky, 1995) which can provide temporal control of transgene expression (St-Onge, Furth, & Gruss, 1996), approaches for genome editing that circumvent constitutive gene deletion have increased the precision of genetic manipulations in the decades since the generation of the first transgenic mouse.

RNA interference—To restrict neuroadaptations both spatially and temporally, viral vectors can be employed to deliver RNA interference (RNAi) or overexpression constructs to neurons. RNAi employs short complementary RNA sequences that, upon binding the desired mRNA, target it for degradation (Paddison, Caudy, Bernstein, Hannon, & Conklin, 2002; Paddison, Caudy, & Hannon, 2002). Virus-mediated RNAi has exposed critical involvement of various neuropeptides, signaling systems and receptors in circumscribed brain regions to regulate alcohol intake and related behaviors.

Viral RNAi has been employed in the alcohol field to study the contribution of alcohol-induced targets to the regulation of alcohol intake, with focus largely on factors expressed in striatal subdivisions and their afferent regions. Viral RNAi confirmed the crucial functions of GDNF in the ventral tegmental area (VTA) (Ahmadiantehrani et al., 2014) and nucleus accumbens (NAc) (Barak et al., 2015) and BDNF in the dorsal striatum (Jeanblanc et al., 2009), to promote moderate drinking, including pinpointing BDNF's effects to the dorsolateral subdivision of the striatum (DLS). RNAi and overexpression constructs also have demonstrated negative regulation of BDNF expression by microRNA 124a in the DLS (Bahi & Dreyer, 2013) and prefrontal cortex (Darcq et al., 2015), making microRNA124a a stimulator of alcohol intake. Viral knockdown has extended our understanding of receptor subunits important for alcohol drinking as well. Quinine-resistant drinking required expression of the N-methyl-D-aspartate (NMDA) receptor subunit 2C in the NAc core (Seif et al., 2013), while manipulation of signaling pathways increasing NMDA receptor subunit 2B activity in the dorsomedial striatum (DMS) promoted excessive drinking (Ben Hamida et al., 2013; Darcq et al., 2014). Specific gamma-aminobutyric acid type A (GABA_A) receptor subunits also modulate alcohol consumption, as RNAi-mediated reduction in GABA_A α 2 levels in the CeA (J. Liu et al., 2011) or GABA_A α 4 or GABA_A δ levels in the medial NAc shell (Nie, Rewal, Gill, Ron, & Janak, 2011; Rewal et al., 2012; Rewal et al., 2009) all reduced alcohol intake. RNAi knockdown of mu opioid receptor expression in the VTA also reduced drinking, whereas inhibition of a midbrain trophic factor called midkine elevated alcohol intake (H. Chen, He, & Lasek, 2017; Lasek, Janak, He, Whistler, & Heberlein, 2007). Together these data highlight multiple receptors involved in promoting alcohol drinking and trophic factors working to limit alcohol consumption whose behavioral functions were confirmed by virus-mediated RNAi. However, conventional RNAi can be labor-intensive in its design and optimization, and it lacks cell type specificity – both challenges that technological advances to regulate gene expression *in vivo* have addressed.

Cre-mediated regulation of gene expression using Flip-Excision (FLEX)

constructs—Similar to cellular restriction mechanisms detailed above for TRAP, confining RNAi expression to specific types is necessary to understand differential functions of gene products in defined neuronal populations. While some level of cellular selectivity may be conferred by choice of promoters, achieving true cell type selectivity via viral expression of genetic constructs under cell-specific promoters has proved challenging (Nathanson et al., 2009). One reliable means for restriction of viral gene expression to specific neuronal populations employs a Cre-driven system containing a Flip-Excision, or FLEX, cassette (Schnutgen et al., 2003), also known as Double-floxed Inverse Orientation, or DIO. Prior to Cre recombination, FLEX cassettes contain the RNAi or overexpression sequence and GFP promoter in an inverted orientation, such that they cannot be expressed, flanked by two pairs of loxP sites. Cre recombinase catalyzes the recombination of the loxP sites, thereby reverting the intervening sequence to the correct orientation to drive expression of both the RNAi or overexpression construct and EGFP. FLEX cassettes provide an important advance in the design of RNAi or other transgene expression, allowing for cellular specificity based on Cre expression patterns, since the FLEX cassette will not express in the absence of Cre recombinase. This technology was recently employed to demonstrate that Fyn, a tyrosine kinase that phosphorylates the NMDA receptor subunit 2B in the DMS upon alcohol exposure to facilitate NMDA receptor activity and promote alcohol drinking (Darcq et al., 2014; Gibb, Hamida, Lanfranco, & Ron, 2011; Wang et al., 2007; Wang et al., 2010), acts specifically in neurons expressing the dopamine D1, but not D2, receptor (Phamluong, Darcq, Wu, Sakhal, & Ron, 2017). This technology also was used in the viral TRAP expression constructs discussed above (Nectow et al., 2015; Nectow et al., 2017). Taken together, these findings demonstrate the power of FLEX to enhance our functional understanding of neuroadaptations regulating alcohol drinking at the cellular level.

In addition to neuronal population specificity in mice and rats with Cre expression under cell type-specific promoters, viral technology also provides the capacity to restrict FLEX gene expression within a defined circuit. Retrograde viral delivery of Cre recombinase in one brain region and viral FLEX infusion in a distinct but synaptically connected cell population will restrict transgene expression to neurons with somas located in the viral FLEX-infused brain region and axon terminals in the retroviral Cre-infused region (Hnasko et al., 2006; Senn et al., 2014). The capacity to activate FLEX-regulated gene expression in a circuit-specific manner via retroviral Cre expression has been demonstrated previously for both optogenetics and chemogenetics (Cheng et al., 2017; Gremel et al., 2016). The ability to elucidate how individual gene products function at the circuit level will greatly enhance our discovery of the exact contribution of individual molecular targets to the regulation of neuronal communication driving alcohol-related behaviors.

Bacterial DNA Editing Technologies: CRISPR/Cas9, TALENs and ZNFs—A final frontier for elucidating the involvement of alcohol-regulated proteins in promoting alcohol consumption and related behaviors is the ability to permanently remove target expression through genome editing in terminally differentiated adult neurons. Recent advances in genetic editing have identified three families of bacteria-derived enzymes that have shown promise as tools for manipulating the molecular composition of both pre-mitotic and post-

mitotic cells: CRISPR/Cas (clustered regularly interspaced short palindromic repeats/CRISPR-associated nuclease), TALEN (transcription activator-like effector nucleases) and ZFN (zinc finger nucleases) (Heidenreich & Zhang, 2016). All three techniques irreversibly modify DNA by targeted DNA cleavage. These mutation strategies provide an advantage over traditional transgenic animals by circumventing developmental anomalies or compensatory changes that may obscure the true function of the deleted gene product. Of these technologies, CRISPR/Cas has been most widely adopted due to the simplicity of the target-specific engineering required: a single guide RNA sequence to direct Cas to the appropriate location in the genome (Staahl et al., 2017). CRISPR/Cas has been utilized to successfully generate knockout mouse and rat lines (Li et al., 2013), including to insert a mutation specific to the alcohol interaction site on the GABA_A $\rho 1$ receptor subunit (Blednov et al., 2017). This minor genetic change produced faster recovery of motor coordination and greater functional tolerance to alcohol in the absence of altered receptor expression levels. CRISPR/Cas9 also has been employed to look at the involvement of chromatin remodeling in GABA_A $\alpha 1$ (*Gabra1*) sensitivity to alcohol (Bohnsack, Patel, & Morrow, 2017). Lentiviral delivery of a Cas9 fusion construct coupling Cas9 to an acetyltransferase, as well as the guide RNA to direct recombination to *Gabra1*, altered the acetylation state of *Gabra1* in cortical primary neurons, conferring protection from alcohol-induced reduction in *Gabra1* mRNA and GABA_A $\alpha 1$ protein expression. Despite these successes, barriers still exist to the widespread adoption of this technology to manipulate adult neurons *in vivo* (Heidenreich & Zhang, 2016). Viral packaging of the Cas9 protein has proved somewhat challenging, although recent reports indicate modified viral strategies for Cas9 delivery show promise for viral CRISPR to permanently alter DNA in adult neurons (Nishiyama, Mikuni, & Yasuda, 2017; Staahl et al., 2017; Tervo et al., 2016). One remaining limitation of this technique is the lack of cell type specificity in current constructs, requiring incorporation of restrictive promoters into the viral expression construct, in the absence of rodent lines expressing Cas9 in circumscribed populations. Mice have been generated with Cas9 expression under the control of Cre recombinase (J. Chen, Du, He, Huang, & Shi, 2017), which allow restricted gene knockout in neuronal subpopulations based on the strategy employed for Cre expression. Expansion of Cas9 expression under Cre regulation to allow for viral Cas9 delivery would achieve the highest level of precision in CRISPR-directed gene deletion. Like other emerging technologies, this shortcoming will likely be overcome within the next few years, which would provide CRISPR/Cas9 with the same precision and flexibility currently afforded to FLEX-RNAi expression. Together these emerging technologies enhance the ability of the alcohol research field to address both cell type- and circuit-restricted involvement of individual molecular targets to regulate the various alcohol-related behaviors, including drinking, that characterize AUD.

Summary

AUD results from many neuroadaptations that may differentially contribute to the various behavioral phenotypes classified as components of AUD. The development of molecular techniques capable of interrogating the molecular bases of AUD with enhanced specificity at the cellular and circuit levels is crucial for continued progress in elucidating precisely how alcohol impacts the brain and the exact pattern of adaptations necessary to generate a

defined set of maladaptive behaviors. Through continued refinement in our understanding of the biochemical basis of this disorder, new treatments may be discovered with enhanced therapeutic success in specific subpopulations of AUD individuals.

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Highlights

- TRAP simplifies sequestration of mRNA targeted for translation
- TRAP can profile mRNA expression by cell types or subcellular compartment
- Viral CRISPR techniques can manipulate gene expression in a restricted fashion
- FLEX can target genetic manipulation by cell type via Cre recombination

Figure 1. TRAP technology enhances the precision of qPCR templates

Extraction of mRNA from brain tissue collected by microdissection of a region of interest without post-dissection processing produces a mixed-cell total mRNA template (top left). Increasing levels of precision in the source of qPCR mRNA templates is seen with progression through the flow chart, highlighting important differences in the interpretation of observed mRNA alterations based on what manner of template – from the heterogeneity of total mRNA to neuronal population-specified ribosome-associated RNA – is quantified.